Chemokine expression and regulation in human gastric epithelial cells: Association with gastritis and H. pylori

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Chemokine expression and regulation in human gastric epithelial cells: association with gastritis and *H. pylori*

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TABLE OF CONTENTS

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ABSTRACT

Chemokine and chemokine receptor expression have been investigated in the gastric mucosa in association with *H. pylori* infection and gastritis.

The AGS and MKN45 gastric epithelial cell lines were cultured in the presence of the pro-inflammatory cytokines IL-1α, TNF-α and IFN-γ added alone or in combination up to 48 hours and were investigated for the expression of IL-8/CXCL8 and RANTES/CCL5 mRNA by Northern analysis. The cytokine-stimulated cell lines were found by RT-PCR to express 'CXC' and 'CC' chemokines including IL-8/CXCL8, MIG/CXCL9, IP-10/CXCL10, SDF-1α/CXCL12, BCA-1/CXCL13, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, MCP-3/CCL7 and LARC/CCL20. The AGS and MKN45 cell lines were found to express IL-8/CXCL8, MIG/CXCL9 and LARC/CCL20 constitutively.

The regulation of IL-8/CXCL8 and RANTES/CCL5 by IL-4 and IL-13 were investigated by Northern analysis and ELISA. Neither IL-4 nor IL-13 modulated the IL-8/CXCL8 production in the AGS and MKN45 cells but both cytokines down-regulated the RANTES/CCL5 secretion in the MKN45 cells.

Chemokine expression was also investigated by RT-PCR in gastric biopsies from patients with *H. pylori*-associated gastritis, non-*H. pylori*-associated gastritis and normal controls. Many 'CXC' chemokines including IL-8/CXCL8, MIG/CXCL9, IP-10/CXCL10, SDF-1α/CXCL12 and BCA-1/CXCL13 and 'CC' chemokines MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5 and LARC/CCL20 were expressed at higher levels in patients with gastritis. The chemokine expression was not totally dependent on the presence of *H. pylori* infection but seemed to be an inflammatory response. Chemokines detected in the gastric biopsies from the normal patients were MCP-1/CCL2 and SDF-1α/CXCL12.

Chemokine receptors were investigated in the AGS and MKN45 cell lines by RT-PCR. MKN45 cells expressed CXCR4 and CXCR5. CCR2 were found to be expressed in both cell lines. The AGS cells also expressed CCR1 and CXCR1.

Immunohistochemical experiments performed on histology slides demonstrated that epithelial cells from normal patients expressed low levels of BCA-1/CXCL13 while patients with *H. pylori*-associated gastritis demonstrated very intense staining for BCA-1/CXCL13 in the epithelial cells.

CXCR4, the SDF-1α/CXCL12 receptor was found to be expressed constitutively by gastric epithelial cells although staining was more dense in epithelial cells from *H. pylori*-infected patients.

These chemokines and their receptors may be important in immunosurveillance and epithelial cell behaviour.

LIST OF FIGURES AND TABLES

ABBREVIATIONS

ANOVA	Analysis of variance
BCA-1	B cell chemoattracting chemokine-1
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CCR	CC chemokine receptor
CD	Clusters of differentiation
cDNA	Complimentary deoxyribonucleic acid
COX	Cyclooxygenase
CSPD	Disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5-chloro) Tricyclo(3.3.1 ^{3,7})decan)-4-yl) phenyl phosphate
CXCR	CXC chemokine receptor
DAB	3,3'Diaminobenzidine
DARC	Duffy antigen receptor for chemokines
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
EC	Entero-chromaffin
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENA-78	Epithelia-derived neutrophil attracting-78
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
HBSS	Hanks Balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HIV	Human immunodeficiency virus
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IFN-γ	Interferon-gamma

IP-10	IFN-γ-inducible –10
LARC	Liver and activation-regulated chemokine
LPS	Lipopolysaccharide
MCP-1	Monocyte-chemotactic protein-1
MIG	Monokine induced by IFN-γ
MIP-1α	Macrophage inflammatory protein-1α
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa B
NSAID	Non-steroidal anti-inflammatory drug
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin A
PMA	Phorbol-12-myristate-13-acetate
RANTES	Regulated on activation, normal T expressed and secreted
RT-PCR	Reverse transcriptase polymerase chain reaction
SDF-1α	Stromal cell-derived factor 1α
SEM	Standard error of the mean
TARC	Thymus and activation related chemokine
TBS	Tris buffered saline
Th	T helper
TNF	Tumour necrosis factor
Tween 20	Polyoxyethylenesorbitan monolaurate

1.0 INTRODUCTION

1.1 BACKGROUND

The stomach is a digestive organ, hence foreign substances including food and microbial pathogens are introduced into the stomach daily. As a result of this, the stomach has a role in food digestion and elimination of the microbial pathogens. Elimination of pathogens is important in order to prevent diseases and pathological states that these organisms may cause. Various types of barriers are found in the stomach to achieve the goal of bacterial elimination, these include the mucus gel barrier, the epithelial cell barrier and the immune barrier (Holzer, 2000).

The bacteria *Helicobacter pylori* (*H. pylori*) was discovered by Marshall and Warren in 1982. It is now well-known that the bacteria is responsible for the pathogenesis of various types of gastrointestinal diseases such as gastritis, peptic ulcers, duodenal ulcers, gastric adenocarcinomas and gastric B cell mucosa-associated lymphoid tissue (MALT) lymphomas (Crabtree, 1996). *H. pylori* infections are very common worldwide, although prevalence in the developed world is now decreasing.

The stomach secretes hydrochloric acid which aids food digestion and creates a region of low pH (pH 1) and it also secretes enzyme containing mucus which make the stomach inhospitable to foreign organisms. However, *H. pylori* can survive beneath the mucus layer on gastric epithelial cells, where it is protected from the gastric acidity. Pro-inflammatory cytokines and 'chemokines' which are chemoattractant cytokines are expressed by the host in an attempt to recruit immune cells which can eliminate the bacteria.

Chemokines are pro-inflammatory mediators typically secreted by leukocytes responsible for various processes such as the recruitment of other white blood cells, lymphoid trafficking, lymphoid organ development, inflammation, Th1 and Th2 development, wound healing, angiogenesis and angiostasis as well as metastasis (Rossi et al., 2000). Subsequently, it was discovered that other types of 'non-immune' cells such as endothelial cells, mesangial cells and fibroblasts could also

express chemokines when stimulated with pro-inflammatory cytokines or bacteria. As a result of that, interest in the epithelial cells as a source of chemokines also grew as the epithelial cells were the cells which separated the host from its external environment and were responsible in host protection.

The cytokines and chemokines which are secreted by the gastric epithelial cells are secreted via the T helper 1 (Th1) type of reactions (D'Elios et al., 1997). As a result of the expression of the pro-inflammatory IL-2, IL-12, TNF-β and IFN-γ cytokines, inflammatory cells such as neutrophils, basophils, T and B lymphocytes, macrophages and natural killer cells are attracted to the site of the bacterial infection. This demonstrates the important communication between gastric epithelial cells and mucosal immune and inflammatory cells in the normal host mucosal defense. Once the bacterial infection is eliminated, the inflammation will resolve, however, if this bacteria is not eliminated, persistent inflammatory cell recruitment leading to chronic inflammation will occur (Bamford, 1999). Interestingly, a Th1 type of reaction is not very effective in eliminating extracellular infections such as the *H. pylori* infection and is more suitable for eliminating intracellular bacterial infections. Therefore, anti-secretory and antimicrobial drugs need to be used to assist the immune response to eliminate *H. pylori* effectively from the host.

This study aims to determine if chemokines and their receptors play a role in the maintenance of gastric epithelial cell physiology. Another aspect which will also be of interest would be to examine the regulation and expression of chemokines secreted in inflammatory states in the gastric mucosa and their relationship to *H. pylori* with a view to the possibility of developing novel therapies using antichemokines to treat *H. pylori* infections. This would overcome the problem of the development of antimicrobial resistance which can occur with the common usage of antibiotics.

1.2 ANATOMY OF THE STOMACH

1.2.1 The stomach

The stomach is located in between the oesophagus and the small intestine and is used for storing and digesting food. Food is churned and mixed with gastric juices consisting of hydrochloric acid, pepsin, intrinsic factor and gastric lipase in the stomach and mucus is secreted to lubricate the process. The digested food is then moved to the small intestine by peristaltic waves achieved by contraction of smooth muscles in the stomach wall. The stomach also is involved in the elimination of pathogens by the secretion of hydrochloric acid, the absorption of vitamin B_{12} by the secretion of intrinsic factor and the limited absorption of water, alcohol and drugs (Thibodeau et al., 1993; Tortora et al., 2000). The stomach also secretes the gastrin hormone which regulates the digestive processes.

The stomach has two curvatures, the lesser curvature and the greater curvature (Fig.1). The greater curvature is 4-5 times longer than the lesser curvature. The stomach can be divided into 4 distinct regions, which are the cardia, fundus, body (corpus) and pylorus. The pylorus can be subdivided into the pyloric antrum (antrum) which connects the pylorus to the gastric body and the pyloric canal which connects the pylorus to the duodenum. The cardia is found on the superior opening of the stomach. The fundus is above the level of the cardiac orifice which is the opening from the oesophagus into the stomach. The body is the large central portion of the stomach below the fundus and the pylorus is a muscle controlled opening that separates the stomach from the small intestine (Williams et al., 1993; Tortora et al., 2000).

1.2.2 The structure of the gastric mucosa

The gastric mucosa which is next to the lumen, consists of the three layers, epithelium, lamina propria and the muscularis mucosa (Fig.2). The epithelial layer is made up of gastric pits which contain gastric glands while the lamina propria

Fig.1 **Gross anatomy of the stomach**

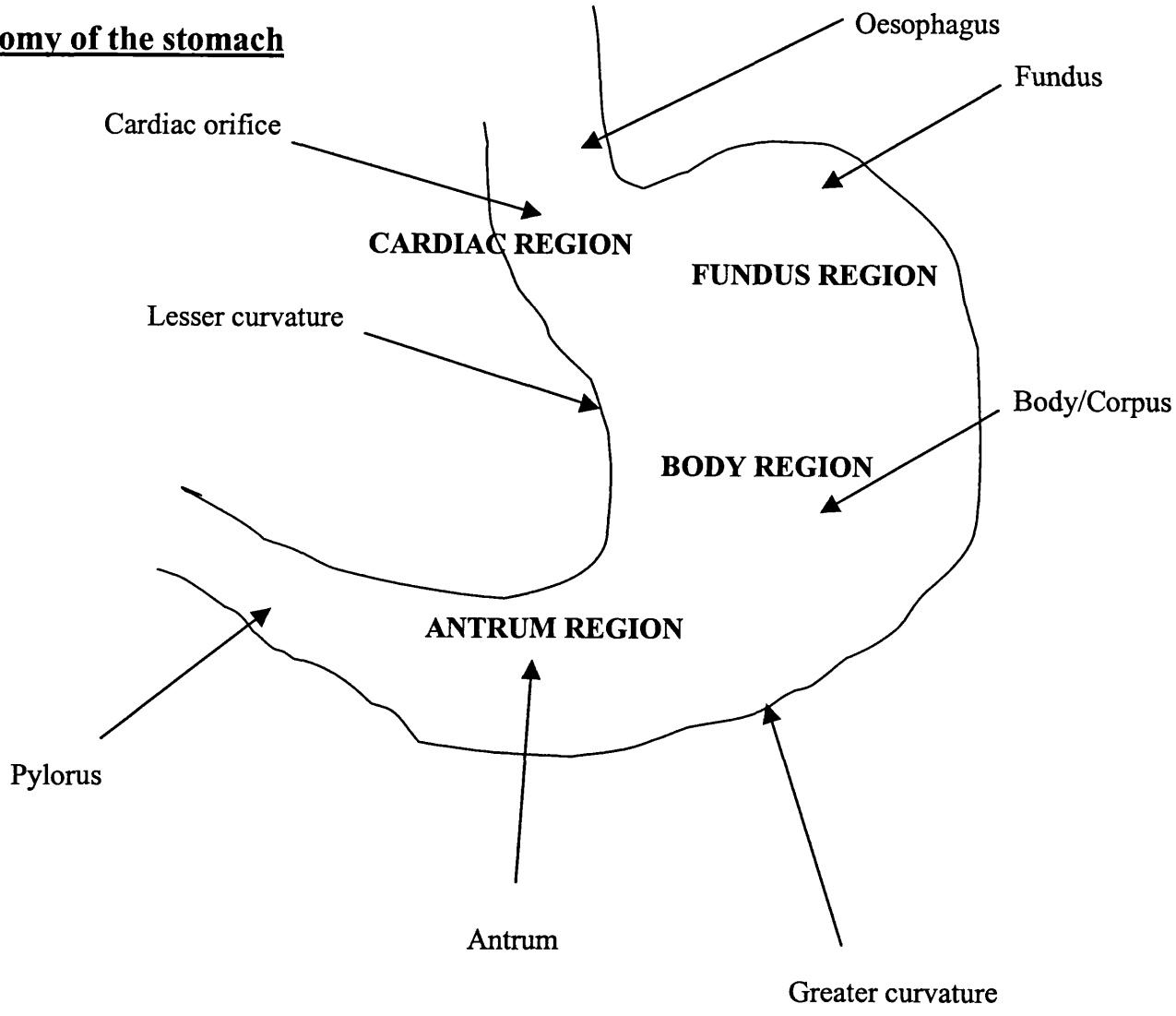
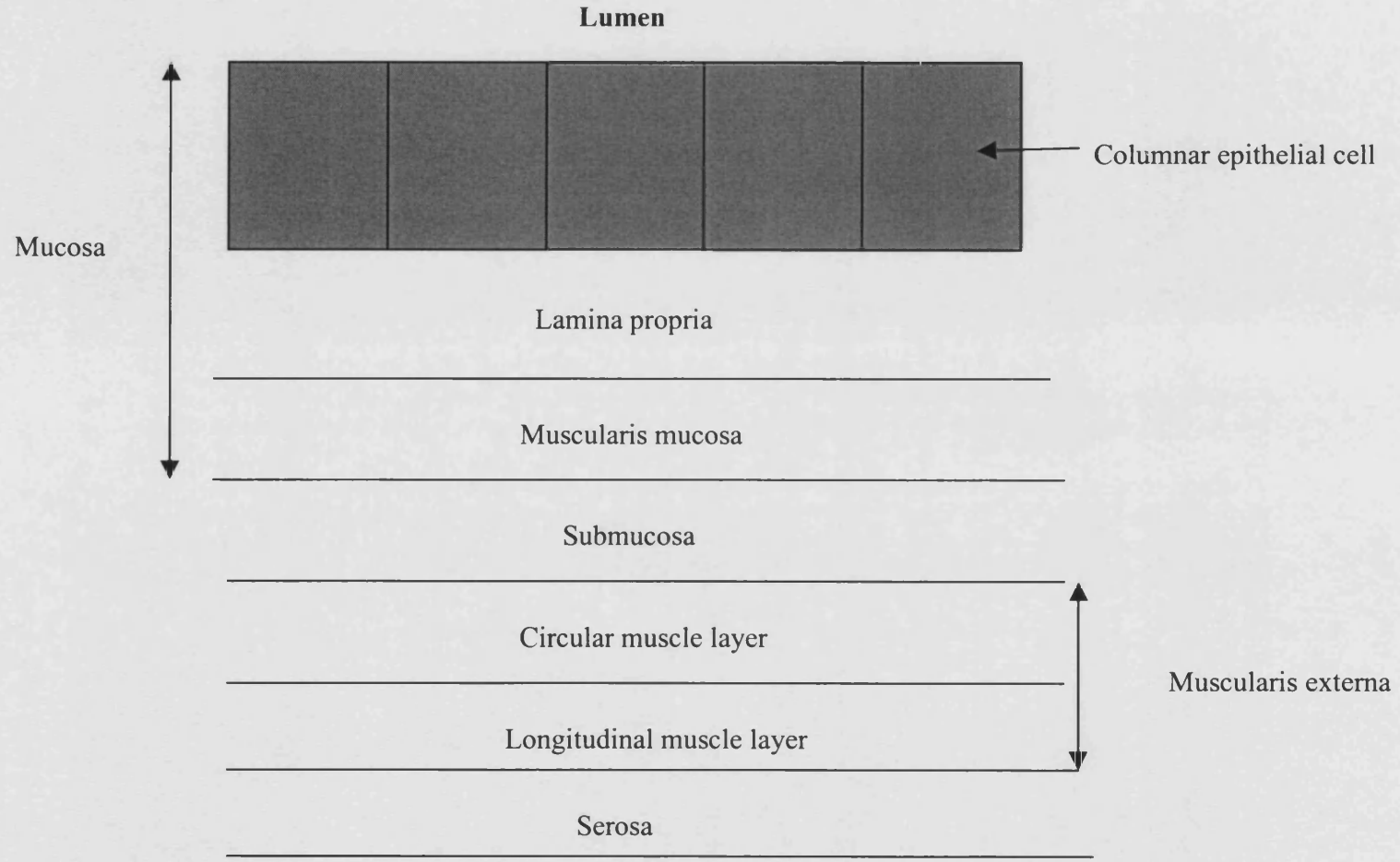


Fig.2 Cross section of a stomach wall



Schematic representation of a stomach wall (adapted from Freeman et al., 1980)

contains reticular, lymphoid connective tissue which functions immunologically and also supports the mucosa. The muscularis mucosa is made up of smooth muscles and is the boundary of the mucosa. Beneath the muscularis mucosa is the submucosa layer which contains arteries, veins, nerves and lymphatics. Under the submucosa layer, there lies the smooth muscle layer made up of the oblique, circular and longitudinal layers of muscularis followed by the serosa layer.

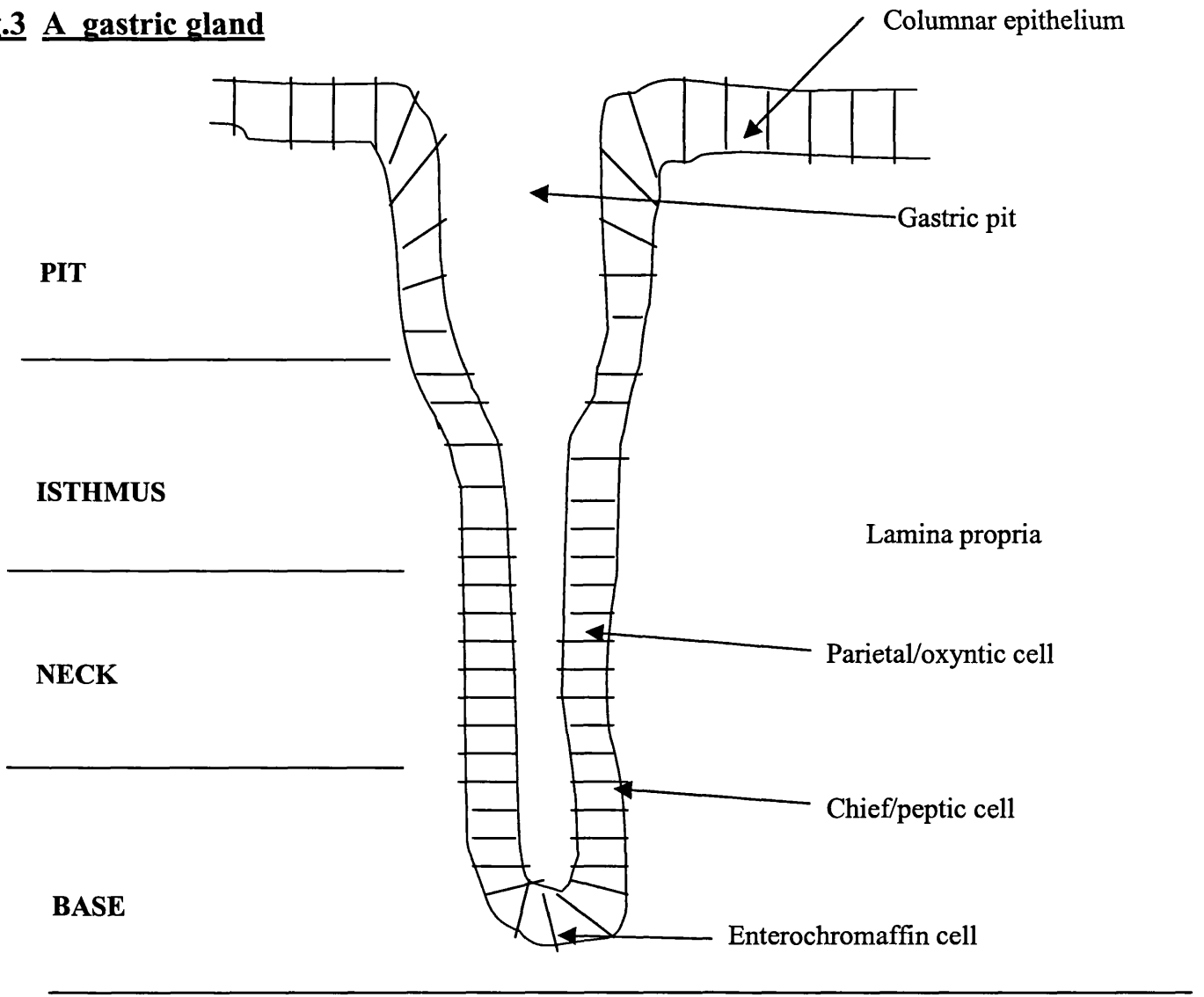
Gastric epithelial cells

The epithelial cells lining mucosal surfaces provide a mechanical barrier which separates the host internal milieu from the external environment (Kagnoff et al., 1997) which may include pathogenic and non-pathogenic micro-organisms. Previously, epithelial cells were assumed to be a passive barrier towards pathogenic organisms and only being a cellular target for such organisms in the worse case. However, in recent years, studies have found that epithelial cells act as sensitive sensors of infection or injury which subsequently initiate defence mechanisms in the form of cytokine or chemokine secretion. Epithelial cells are in an ideal position for this function (Stadnyk, 1994).

The gastric epithelium forms a highly organised layer which covers the free surfaces of the body. The apical surface is the free surface of the cell and the basal surface rests on a basement membrane (Stadnyk,1994). Therefore, gastric epithelial cells play a major role in providing protection to gastric tissues from external irritants, secrete hormones and enzymes which aid in digestion, act as detectors of foreign particle invasion and trigger the immune system to combat the infection or inflammation. Sometimes the responses may become excessive and this leads to inflammatory conditions which provide the basis for various pathological diseases in the stomach.

Gastric pits which consist of gastric glands (Fig.3), occupy the luminal surface of the mucosa. The main gastric glands are located in the fundus and body parts of the stomach and consist of highly differentiated cells with at least 4 cell types and undifferentiated cells (Williams et al., 1989).

Fig.3 A gastric gland



The cells in the main gastric glands are:

i) mucus 'neck' cells
ii) parietal/oxyntic cells
iii) chief/peptic/zymogenic cells
iv) entero-endocrine/argentaffin cells
v) undifferentiated columnar cells

i) Mucus neck cells

These cells are found at the necks of the gastric glands. Their role is to secrete mucus which aids in food lubrication, transports hydrogen and bicarbonate ions and prevents mechanical damage to the surface of the gastric mucosa (Ye et al., 1997).

ii) Parietal/oxyntic cells

These cells are large, round, eosinophilic and have a characteristic 'fried egg' appearance. They are mostly found on the side walls of the gastric glands and near their ducts. Parietal cells have a large, round, central nucleus and very dense mitochondria packed closely together in the cytoplasm. The function of the parietal cells are to secrete chloride and hydrogen ions in the acid secretions of the stomach as well as a protein called intrinsic factor which is needed for vitamin B_{12} absorption.

ii) Chief/peptic/zymogenic cells

These cells are mostly found in the basal part of the gastric gland and appear cuboidal in shape. Unlike the parietal cells, they contain many secretory vacuoles and have a lot of granular endoplasmic reticulum and a distinct Golgi complex. These cells function to produce gastric digestive enzymes such as pepsinogen which digests protein.

iv) Entero-endocrine/argentaffin/enterochromaffin cells

These cells are found in all types of gastric glands but more so in the body and fundus. They can usually be found in the deeper parts of the gastric gland between the chief cells and the basal lamina or lamina propria into which they discharge their products. They have irregular nuclei with a granular cytoplasm and a well-developed rough endoplasmic reticulum and Golgi membranes. These cells produce peptide hormones and vasoactive amines in addition to the peptides gastrin and somatostatin. Gastric motility is increased by gastrin which also increases the secretion of hydrochloric acid and pepsinogen. Somatostatin can inhibit the function of gastrin and other hormones. Insulin and glucagon producing cells are also found in the stomach which are directly involved in the regulation of insulin. Histamine, which stimulates gastric secretions and increases vascular permeability is also secreted by the entero-endocrine cells together with a hormone serotonin which can inhibit the role of histamine.

v) Undifferentiated columnar cells

The low number of undifferentiated cells appear to be the stem cells which undergo cell division and differentiation which replace the other cell types in the gastric glands.

The gastric glands differ slightly in their composition depending on their position, whether they are in the cardiac, main or pyloric parts of the stomach. In different locations in the stomach, the gastric gland differs slightly in its cellular composition. At the proximal and distal ends of the stomach, there are mostly mucus cells. The gastric pits and glands in the cardiac region are short, tubular glands whereas in the pyloric region, the glands are longer, coiled and more branched. No parietal or chief cells are present in the cardiac or pyloric regions of the stomach. The entero-endocrine cells are mostly found in the pyloric region (Borysenko et al., 1989; Williams et al., 1993).

The stomach can be divided into 3 histological regions which are the cardia, body and antrum.

- **Cardiac mucosa**

The cardia is located in the first part of the stomach below the part where the oesophagus joins the stomach. It is made up of simple tubular or cystic glands lined by mucus secreting cells. There are also many endocrine cells which secrete hormones and a few scattered parietal and chief cells (Dixon, 1996).

- **Body mucosa**

The part of the stomach defined histologically as the body mucosa occupies the proximal two-thirds of the stomach and is made up of tightly-packed tubular glands. The upper parts of the glands comprise of parietal cells and the lower parts of the gland are made up of chief cells. There are also mucus neck cells at the bases of the gastric pits and endocrine cells which are also called enterochromaffin cells (Dixon, 1996).

- **Antral mucosa**

Antral mucosa is found in a triangular part proximal to the pylorus in the last part of the stomach. The glands in the antral mucosa are more branched, tortuous and less tightly packed than the body mucosa. There are mucus-secreting cells, endocrine cells and parietal cells lining the glands. The endocrine cells secrete several types of hormones. The G cells secrete gastrin, the D cells secrete somatostatin, the EC (enterochromaffin) cells secrete 5-hydroxytryptamine, 5-HT, the P cells secrete bombesin and the S cells secrete secretin (Dixon, 1996; Calam, 1999).

1.3 GASTRIC SECRETIONS

The mucus secreted by the columnar mucus-secreting cells on the surface of the gastric mucosa and its pits, together with the mucus from the antral mucus glands create a viscid gel called the gastric mucus barrier which covers the gastric mucosa. The surface epithelial cells also secrete bicarbonate and sodium ions which diffuse into the gel, buffering the hydrogen ions which enter from the luminal part of the

stomach. As a result of this, a pH gradient is achieved, whereby the pH is 1 or 2 at the luminal surface of the barrier and neutral at the epithelial plasma membrane (Dixon, 1996).

Food digestion is achieved by the release of hydrochloric acid and pepsin from the parietal and chief cells respectively. These gastric secretions are regulated by the hormones gastrin and somatostatin as well as the vagal nerve system. Gastrin, released from the antral G cells stimulates the parietal cells directly to release gastric acid and indirectly by stimulating the enterochromaffin cells in the gastric body, near the parietal cells to release histamine (Kandel, 2000). Conversely, somatostatin released by the D cells in the gastric antrum antagonises the effect of the gastrin hormone. An overproduction of gastrin, due to the presence of *H. pylori* or the Zollinger-Ellison disease results in an overproduction of gastric acid resulting in gastric ulcers.

Other hormones secreted by the gastric cells are 5-hydroxytryptamine, 5-HT which is secreted by the enterochromaffin cells, bombesin which is secreted by the P cells and secretin which is secreted by the S cells (Dixon, 1996).

1.4 THE IMMUNE SYSTEM

When a foreign bacteria or particle invades the body, the body's immune system is triggered into action to dispose of the foreign bacteria or particle. The immune mechanisms can be divided into:
a) the innate/natural/native immunity and
b) the specific/adaptive immunity

a) The native/innate/natural immunity

The native immunity is the first line of defence against microbes and recognizes particles which are foreign to the host. Subsequently, it launches an attack against the invader. This type of immunity is limited in diversity and functions in the same way against most microbes without distinguishing one microbe from the other. It is very

efficient, however, some organisms have developed mechanisms to evade the innate immunity. Therefore, the innate immunity can also induce the specific immune responses to eliminate the microbes. The components of the innate immunity are:

i) physical and chemical barriers such as epithelia and antimicrobial substances produced at the epithelial surfaces such as defensins

ii) blood proteins including the complement system and mediators of inflammation

iii) phagocytic cells such as neutrophils, macrophages and other leukocytes such as natural killer cells

(Abbas et al., 1997; Eales, 1997; Staines et al., 1993).

b) **The specific/adaptive immunity**

The specific/adaptive immunity is more highly evolved and is triggered by exposure to infectious agents. The magnitude and defensive capabilities of the specific immunity increases with each exposure to a particular microbe. This is called an immunologic memory.

The specific immune system is made up of B lymphocytes, T lymphocytes and antibodies which are the products of B lymphocytes. The T lymphocytes can be subclassed into helper T lymphocytes, cytotoxic lymphocytes and natural killer cells.

B lymphocytes produce antibodies for the humoral immunity. T helper lymphocytes stimulate B cell growth and differentiation and activate macrophages by secreted cytokines.

Helper T lymphocytes can be subdivided again into Th1 and Th2 cells depending on the types of cytokines produced by the activated Th cells (Wells et al., 1998). T suppressor cells suppress immune responses whereas Th2 cells help B cells to make antibodies by synthesising and secreting cytokines which activate the growth and

differentiation of B cells. Th1 produce cytokines such as interleukin-2 (IL-2), tumour necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-12 (IL-12). Th2 cells secrete interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10) and interleukin-13 (IL-13) (Wells et al., 1998). Th1 cytokines are implicated in rheumatoid arthritis and delayed-type hypersensitivity reactions while Th2 cytokines are involved in allergic inflammatory diseases such as asthma and atopic dermatitis.

1.5 CYTOKINES

Cytokines are a group of highly potent, low molecular weight (8-25 kDa) proteins which can mediate the growth of cells, inflammation, immunity, differentiation and repair. Most cytokines are secreted soluble and bind to specific receptors (Stadnyk, 1994). Cytokines usually act locally but may have autocrine and paracrine roles. Cytokines usually have cascade and network effects on the expression of other cytokines, therefore, even low concentrations of cytokines may result in amplified results and effects. Cytokines can be classed into different groups:

i) interleukins
ii) colony stimulating factors
iii) tumour necrosis factors
iv) chemokines
v) others

Cytokines are produced briefly during the activation and effector phases of innate and specific immunity and mediate and regulate immune and inflammatory responses. They are produced by diverse cell types such as by immune cells including lymphocytes, B cells, macrophages, natural killer cells, T cells, monocytes and non immune cells such as fibroblasts, endothelial cells, astrocytes and epithelial cells. They act on many cell types (pleotropic) but may also have different effects on the same cell. IL-1 and TNF-α are examples of pleiotropic cytokines which have many similarities in the biological activities which they induce. Many cytokines usually have redundant and overlapping actions (Staines et al., 1993).

The expression of cytokines by gastric epithelial cells during inflammatory conditions and in disease such as in patients infected with the bacteria *H. pylori*, suggests that there is an interaction between epithelial cells and the immune system and that cytokines act as communication molecules between these cells. Therefore, there is interest in controlling the expression of cytokines and chemokines by the epithelial cells in order to control inflammatory diseases.

1.5.1 Interleukin-1 (IL-1)

Interleukin-1 (IL-1) is a mediator of the host inflammatory response in innate immunity. The activated mononuclear phagocyte (monocyte) is the major cellular source of IL-1. Bacterial lipopolysaccharide (LPS), macrophage-derived cytokines such as IL-1 or TNF-α and $CD4^+$ T cells can trigger the production of IL-1. IL-1 can also be produced by many types of cells such as epithelial cells, fibroblasts, astrocytes, B cells and endothelial cells, although most of it is produced by macrophages (Kolios et al., 1995) and lymphocytes (Stadnyk, 1994). There are 2 forms of IL-1 which are IL-1α and IL-1β. Both of them are 17 kDa polypeptides but share very little homology with each other, although they both have identical biological activities. This is since they both bind to the same IL-1 receptor types (Stadnyk, 1994). IL-1 does not activate inflammatory neutrophils directly but stimulates the synthesis of chemokines by mononuclear phagocytes and endothelial cells which activate leukocytes. In larger quantities, the expression of IL-1 can exert endocrine effects such as fevers, the synthesis of acute phase plasma proteins by the liver and induce metabolic wasting/cachexia.

1.5.2 Tumour necrosis factor (TNF-α)

Tumour necrosis factor-α (TNF-α) is the main mediator of the response to gram-negative bacteria and other infectious organisms. TNF-α is secreted by a number of cell types including monocytes, antigen stimulated T cells, activated natural killer (NK) cells, normal B cells, B-lymphoblastoid cells and activated mast cells. Other 'non-immune' cells that produce TNF-α are fibroblasts, smooth muscle cells, astrocytes, osteoblasts, retinal pigment epithelial cells, gastric epithelial cells

(Nakachi et al., 2000) and others (Aggarwal et al., 1996). TNF-α mediates the innate and specific immunity and links the specific immune responses with acute inflammation. TNF-α is 17 kDa cytokine (Van Deventer, 1998) and in small quantities, it can act as a paracrine and autocrine regulator of leukocytes and endothelial cells. Inducers of TNF-α include gram-positive and negative bacteria, cytokines (IL-1, IL-2, IFN-γ) and others (Le et al., 1987; Larrick et al., 1988). TNF-α can be down-regulated by cytokines such as interferon-α and β (IFN-α/β), IL-4, IL-6, IL-10, transforming growth factor-β and granulocyte colony stimulating factor. TNF-α is also down-regulated by viruses such as the Epstein-Barr virus, and adenovirus proteins and drugs such as cyclosporin A and dexamethasone among others. Some of the functions of TNF-α are to stimulate mononuclear phagocytes and other cells to secrete chemokines which recruit leukocytes, to activate the killing of microbes by inflammatory leukocytes and to stimulate the secretion of IL-1 and IL-6 into the circulation by mononuclear phagocytes and vascular endothelial cells.

TNF-α has been demonstrated to play a role in normal physiological processes such as haematopoiesis, inflammation, reproduction and protection against infection. It also has a clinical role in cancer chemotherapy, when used in conjunction with interferon-γ (IFN-γ) and chemotherapeutic drugs (Aggarwal et al., 1996). However, most of its actions are proinflammatory (Ebert, 1998). TNF-α increases the inflammation by inducing IL-1 production (Le et al., 1987).

In patients with *H. pylori* infection, TNF-α levels were higher in the gastric mucosa than in patients who were *H. pylori* negative (Yamaoka et al., 1996; Yamaoka et al., 1997). $CagA^+$ strains of *H. pylori* induced more cytokines compared to the $CagA^-$ strain (Peek et al., 1995).

1.5.3 Interferon

Interferon (IFN) is a group of polypeptides of 18 kDa. IFN is mostly produced by mononuclear phagocytes (Abbas et al., 1997) and activated Th1 lymphocytes (Warhurst et al., 1998), B cells, epithelia, fibroblasts as well as natural killer cells (Kolios et al., 1995). There are three forms of interferons which are IFN-α, IFN-β

and IFN-γ although IFN-γ does not show significant homology to the other two IFNs. IFN-γ synthesis is inhibited by vitamin D3, dexamethasone and Cyclosporin A, conversely IL-2, basic fibroblast growth factor (BFGF) and endothelial growth-factor (EGF) induce the synthesis of IFN-γ (De Maeyer et al., 1992; Farrar et al., 1993). IFN-γ acts in synergy with other pro-inflammatory cytokines for example, TNF-α to stimulate the secretion of chemokines in various systems (Warhurst et al., 1998). IFN-γ has antiviral and antiparasitic properties while also inhibiting the proliferation of some normal and transformed cells. However, immunomodulation seems to be the main biological activity of IFN-γ. It is moderately useful when used in conjunction with other IFNs and chemotherapy to treat cancer since it inhibits cell proliferation (Kardamakis, 1991; Ernstoff et al., 1992; Schiller et al.,1992). This cytokine is used to regulate the Th1 and Th2 responses in activating mononuclear cells (Sawai et al., 1999) which control infections in the long-term. IFN-γ is not secreted by epithelial cells during infections. During an infection, pathogens stimulate epithelial cells to secrete cytokines, chemokines and stress proteins that attract neutrophils. The stress proteins stimulate the lymphocytes to secrete IFN-γ, IL-2, IL-4, IL-9 and IL-13 around the epithelial cells which stimulate the expression of adhesion molecules on the epithelial cell. The neutrophils then bind to the adhesion molecules on the epithelial cells and ingest the foreign organisms (Strober, 1998). IFN-γ is involved in gastric inflammation caused by *H. pylori* infection although it might also have a role in protection from *H. pylori*. The suggested protective mechanism of IFN-γ is to increase the intensity of the antigen-presenting cell function of gastric epithelial cells (Sawai et al., 1999) possibly through the up-regulation of the class II major histocompatibility complex (Ye et al., 1997).

1.5.4 Interleukin-4 (IL-4)

IL-4 is a 20kDa cytokine and is a member of the 4-α-helical cytokine family. It regulates the IgE and mast cell/eosinophil-mediated immune reactions. IL-4 is produced by $CD4^+$T lymphocytes specifically of the Th2 subset, mast cells, basophils and $CD8^+$ T cells.

The actions of IL-4 are:

a) to aid in the production of IgE and is the main cytokine that stimulates the switching of B cells to this heavy chain isotype
b) as a factor for growth and differentiation for T cells, especially for the Th2 subset
c) to stimulate the expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells increasing the binding of lymphocytes, monocytes and eosinophils
d) as a growth factor for mast cells and to stimulate mast cell proliferation together with IL-3

IL-4 inhibits the production of inflammatory cytokines such as IL-1, IL-6 and TNF-α by monocytes and TNF-α by T cells. Therefore, it is useful in the regulation of inflammatory diseases and autoimmune diseases. IL-4 also inhibits the growth of colon and mammary tumours, hence it may also be used in the treatment of solid tumours (Dullens et al., 1991, Toi et al., 1992).

1.5.5 Interleukin-13 (IL-13)

Interleukin-13(IL-13) is a recent addition to the T helper (Th2) family of cytokines. IL-13 is distantly related to IL-4 as they both belong to the α-helix family of proteins and they both share some similar structural characteristics (Brombacher, 2000). IL-13 is produced by activated T lymphocytes, mainly Th2 cells, mast cells, basophils, dendritic cells and natural killer cells (Zurawski et al., 1994). It is not expressed in the heart, brain, placenta, lung, liver and skeletal muscle tissues. IL-13 has immunomodulatory effects on a variety of different types of cells such as monocytes, macrophages, natural killer cells, fibroblasts, eosinophils, airway smooth muscle cells and endothelial cells. IL-13 could potently suppress the cytokine and chemokine generation by activated monocytes and macrophages (Minty et al., 1993; Zurawski et al., 1994) and endothelial cells (Marfaing-Koka et al.,1995). It has also been shown to up-regulate some of the integrin superantigen family (adhesion molecules) which induce the trafficking of these cells. IL-13 has both immunosuppressive and anti-inflammatory properties (Minty et al., 1993). Some of

the cytokines that IL-13 are known to inhibit include IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, GM-CSF, G-CSF and chemokines such as IL-8/CXCL8, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, MCP-3/CCL7 and eotaxin/CCL11 in response to IFN-γ or bacterial lipopolysaccharides. Due to their immunomodulatory and anti-inflammatory properties, IL-13 and IL-4 show great promise to be used in the treatment of chronic inflammatory diseases.

1.5.6 The effect of IL-4 and IL-13 on cytokines secreted by cell lines

It has been suggested that IL-4 and IL-13 are anti-inflammatory cytokines. The inhibitory effect of IL-13 could be a result of enhancement of the IL-1 receptor antagonist (IL-1ra) and soluble receptors and a direct inhibition of chemokine transcription (Muzio et al., 1994). However, the converse was found to be true in some types of cells. Therefore, IL-13 has a bifunctional role depending on the stimuli and chemokines being investigated (Kolios et al., 1996).

1.6 CHEMOKINES

Chemoattractant cytokines, collectively known as chemokines are a family of low-molecular weight proteins (8-14 kDa), consisting of 70-100 amino acids (Baggiolini et al., 1994; Horuk et al., 1994; Rollins, 1997; Balkwill, 1998, Nagasawa et al., 1998; Pelchen-Matthews, 1999). Traditionally, the main known role of chemokines was to attract the migration of many types of leukocytes including neutrophils, T cells, monocytes, natural killer cells, basophils, eosinophils and mast cells to the sites of inflammation or infection. This was achieved by the activation of cell adhesion molecules which regulate the activation and movement of leukocytes (Zack Howard et al., 1996). Leukocyte migration is important for immune surveillance of the tissues of the body and for focusing immune cells to the site of the antigenic challenge. Leukocyte migration is dependent on the actions of various adhesion molecules, chemokines and their receptors (Qin et al., 1997; Sallusto et al., 1998).

Different chemokines attract different leukocytes. Leukocytes travel to the site of infection or injury in the blood. Leukocytes roll along the endothelial cells which make up the walls of the blood vessels (Fig.4). When the leukocytes reach the site of infection or injury, intercellular adhesion molecules (ICAM-1/CD54) are expressed on the surface of the endothelial cells as well as the leukocytes which enable the leukocytes to attach themselves to the endothelial cells. Following this, the leukocytes migrate or enter the site of injury or infection by squeezing in between the endothelial cells. These leukocytes then attach themselves via the adhesion molecules(ICAM-1/CD54) expressed on the gastric epithelial cells and cause inflammatory processes to occur at the gastric mucosa (Crowe et al., 1995).

Some examples of specific chemokines which attract leukocytes are SDF-1α/CXCL12 which attracts most peripheral blood lymphocytes and LARC/CCL20 attracts memory $CD4^+$ T cells (Campbell et al., 2000). Apart from inducing inflammatory conditions by attracting leukocytes, chemokines may also have other basic functions in the immune system which are not directly related to the inflammatory processes. These other functions include regulation in the trafficking of immature blood cells and naïve lymphocytes (Chang et al., 1999; Kim et al., 1999).

It is now known that chemokines are not only expressed by 'immune' cells such as monocytes, macrophages, dendritic cells but can be expressed by other types of 'non-immune' cells such as fibroblasts, epithelial cells, stromal cells from many sources, keratinocytes, chondrocytes, mesangial cells, endothelial cells and smooth muscle cells (Baggiolini et al., 1994; Luster, 1998; Kim et al., 1999). These cells produce chemokines when they are subjected to the appropriate conditions and stimulations and these chemokines are expressed constitutively or in an inducible manner. Constitutively expressed chemokines are important for cell homing and the trafficking of leukocytes under normal conditions, whereas inducible chemokines mediate inflammatory and immune responses (Sallusto et al., 1998; Sozzani et al., 1999).

More recently, it has been realised that chemokines had effects on cells other than leukocytes. Chemokines may affect the migration and proliferation of endothelial cells which is important in angiogenesis and angiostasis and may also affect the

Fig.4 A diagram of leukocyte migration to the site of injury/infection

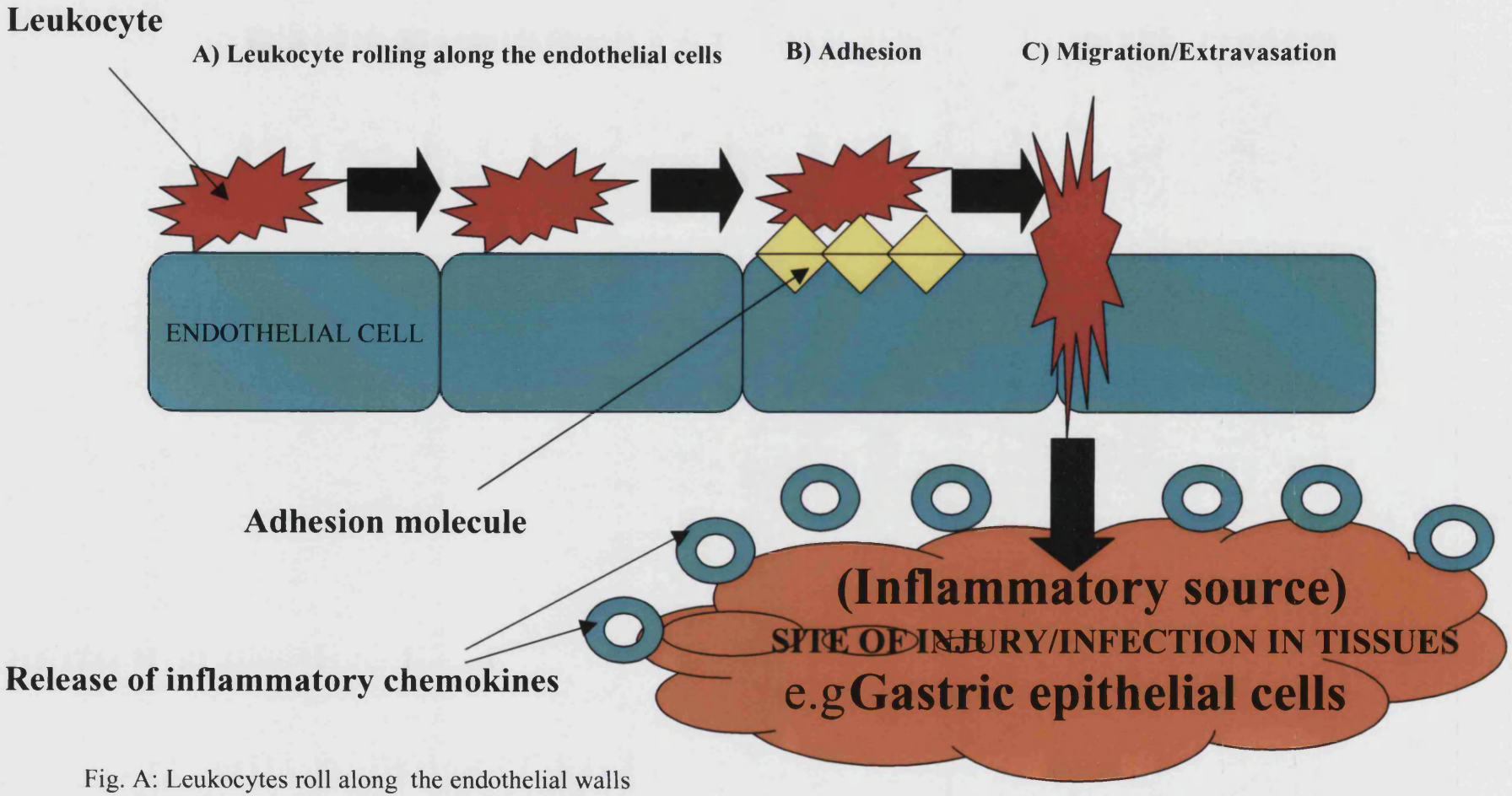


Fig. A: Leukocytes roll along the endothelial walls
B: Adhesion molecules (eg. ICAM-1, VCAM-1) tether the leukocytes to the endothelial walls
C: Leukocytes migrate to the site of injury/infection

migration of smooth muscle cells which may be important in atherosclerosis (Stemme & Hansson, 1994). The responses induced by the chemokines include chemotaxis, release of enzymes from intracellular stores, formation of oxygen radicals, a change of shape through cytoskeletal rearrangements, generation of lipid mediators and adhesion to endothelium or extracellular matrix protein.

The main biological activities of chemokines include directing the movement of leukocytes, angiogenesis and angiostasis, Th1/Th2 development, wound healing, cell recruitment, lymphoid organ development and haematopoiesis. Apart from a role in immunoregulation, chemokines are also involved in inflammatory and disease states (Schall et al., 1994) such as in inflammation and tumour metastasis. Examples of inflammatory diseases that are associated with the expression of chemokines are glomerulonephritis (Brown et al., 1996), rheumatoid arthritis (Koch et al., 1992), asthma (Teran et al., 1996) and inflammatory bowel disease (Grimm et al., 1996). During inflammation, chemokines have been detected in most organs and in many types of cells. Therefore, many types of cells can secrete the chemokines in response to the appropriate stimuli such as pro-inflammatory cytokines including IL-1 and TNF, bacterial lipopolysaccharides (LPS) and viral infections, IFN, IL-4 and Th1 and Th2 products (Strieter, 1996; Luster, 1998). Conversely, chemokine expression can be down-regulated by anti-inflammatory cytokines such as IL-10 (Karakurum et al., 1994), IL-4 and IL-13 (Kolios et al., 1999).

IL-8/CXCL8 and IP-10/CXCL10 modulate the proliferation and migration of smooth muscle cells, on the contrary, MCP-1/CCL2 increases the proliferation of smooth muscle cells (Xu et al., 1996) and upregulates intercellular adhesion molecules (ICAM-1). MCP-1/CCL2 also encourages coagulation activities which stimulate the formation of thrombosis in atherosclerotic plaques (Yla-Herttuala et al., 1991). SDF-1α/CXCL12 also is a chemoattractant in glial and endothelial cells (Murdoch, 2000).

There are 4 main chemokine subfamilies which are the 'CXC' (α), 'CC' (β), 'C' (γ) and 'CX_3C' (δ) families (Shimada et al., 1998; Sato et al., 1999). This distribution of chemokines into the various families is based on the 4 cysteines (represented by the letter 'C') in the terminal amino section of the chemokine molecule which are linked

by disulphide bonds (Bliss et al., 1998) to the more structured core of the molecule (Mantovani, 1999). The exception to this rule is the chemokine which belongs to the 'C' family which is called lymphotactin which only has 2 cysteines. The letter 'X' represents an amino acid (Fig.5).

1.6.1 CXC chemokines

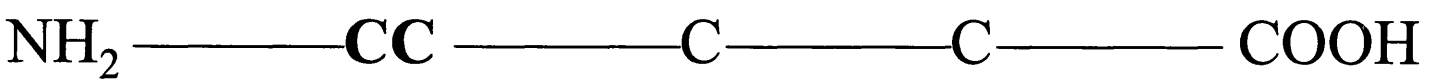
These chemokines have 4 cysteine residues which are separated by a single amino acid (Schrum et al., 1995; Van Deventer, 1997). The 'CXC' (α) chemokines can be further subdivided into those which contain the glutamic acid-leucine-arginine ('ELR') sequence between the N-terminus and the first cysteine and those which do not (Rollins, 1997; Zlotnik et al., 2000). The 'ELR'-containing 'CXC' chemokines exert angiogenic effects while the non-'ELR' 'CXC' chemokines exert angiostatic effects (Strieter et al., 1995). However, there is an exception to this rule, whereby SDF-1α/CXCL12, a non-'ELR' 'CXC' chemokine exhibits angiogenic activity (Schnyder-Candryan et al., 1995; Romagnani et al., 2001). The 'ELR' 'CXC' chemokines have an ability to bind to a shared chemokine receptor, CXCR2, (Schall et al., 1994) conversely the 'CXC' chemokines without the 'ELR' motif bind to the receptors CXCR3, CXCR4 and CXCR5 (Zlotnik et al., 1999). These chemokines are produced by activated mononuclear phagocytes, tissue cells such as endothelial cells, fibroblasts and megakaryocytes and typically attract neutrophils (Baggiolini et al., 1994; Shimoyama et al., 1994; Raport et al., 1996) in acute inflammatory conditions. The 'CXC' chemokines without the 'ELR' motif attract T lymphocytes (Mantovani, 1999). 'CXC' chemokines are associated with acute inflammatory states such as acute respiratory distress syndrome and septic shock (Wells et al., 1998). IL-8/CXCL8 is the "prototype" CXC chemokine and was the first one to be identified. Many studies have been performed to study this chemokine.

Fig.5 Schematic diagrams of chemokine families

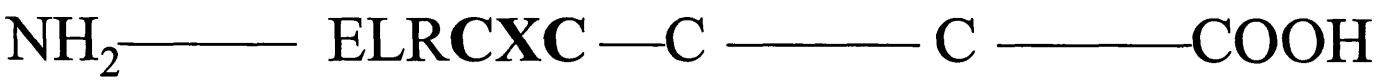
C = cysteine
X = any amino acid
E = glutamic acid (specific amino acid)
L = leucine (specific amino acid)
R = arginine (specific amino acid)

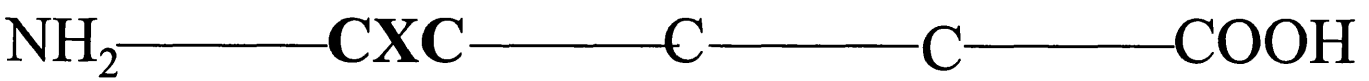
NH2 = amino
H = hydrogen
O = oxygen

I) **CC chemokine family**

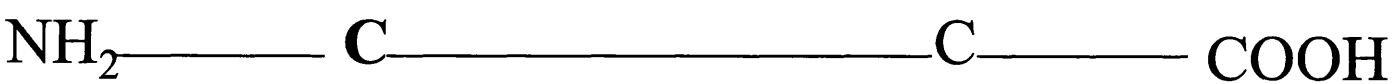


II) **CXC chemokine family**

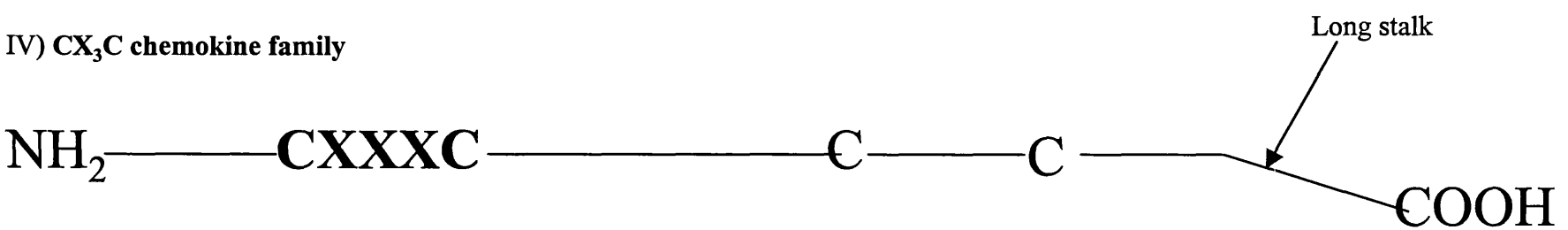




III) **C chemokine family**



IV) **CX_3C chemokine family**



1.6.2 CC chemokines

In the 'CC' (β) chemokine family, the cysteine residues are adjacent to one another (Schrum et al., 1995; Van Deventer, 1997). The 'CC' chemokines are produced by T cells which have been activated and they stimulate eosinophils, basophils, monocytes, dendritic cells, natural killer cells and T cells (Raport et al., 1996; Mantovani, 1999). The 'CC' chemokines do not act upon neutrophils. These chemokines are associated with chronic inflammatory diseases such as asthma, arthritis and atherosclerosis (Wells et al., 1998).

Recently, novel 'CC' chemokines such as TARC/CCL17 and LARC/CCL20 have been discovered. They have features which distinguish them from the traditional 'CC' chemokines which are their constitutive expression in some tissues, specificity for lymphocytes, interaction with receptors highly specific for each chemokine and the chromosomal gene loci different from the traditional 'CC' chemokine gene cluster (Yoshie et al., 1997).

1.6.3 C chemokines

This subfamily of chemokines is also known as the γ family (Gale et al., 1999). This family lacks the first and third cysteines in the typical chemokine structure and has only 2 cysteine residues (Rossi et al., 2000). It has two members which are lymphotactin (XCL1) and SCM-1β (XCL2). Lymphotactin is chemotactic for lymphocytes (Shimoyama et al., 1999), B and T cells and natural killer cells but it does not attract neutrophils or monocytes (Schall et al., 1994).

1.6.4 CX_3C chemokines

The fourth chemokine subfamily is the 'CX_3C' (δ) family which has 3 amino acids in between the 2 cysteines in the amino terminal. An example of the chemokine in this family is fractalkine, also called neurotactin. This chemokine attracts monocytes,

natural killer cells and T cells (Bazan, 1997; Mantovani, 1999). Fractalkine is the only chemokine to be attached to the membrane by a mucin-like stalk (Mahalingam et al., 1999; Zlotnik et al., 2000).

1.7 CHEMOKINE NOMENCLATURE

Recently, a new nomenclature system for chemokines was proposed by Dr Osamu Yoshie (Kinki University, Japan) at the 1999 Keystone Conference. This has now been agreed by the other international chemokine experts (Zlotnik et al., 2000). This nomenclature was proposed to minimise the confusion associated when a chemokine was given a variety of different names. This new nomenclature is based on the previous chemokine receptor nomenclature except where 'L' (for ligand) is used instead of 'R' (for receptor). The numbering system used in the new nomenclature is the same one as that presently used to specify the genes encoding each chemokine. This new nomenclature system also shows the subfamily where each chemokine belongs.

In this section, the chemokines which have been studied in the reverse-transcription polymerase chain reaction (RT-PCR) experiments are explained in more detail. The expression of these chemokines were examined in the AGS and MKN45 gastric epithelial cell lines as well as in human gastric biopsies taken from patients with different pathological states.

Table 1. Nomenclature of chemokines (adapted from Zlotnik et al., 2000).

New systematic name of chemokine	Former name of chemokine	Chemokine receptor(s)	Cells chemoattracted or activated by chemokine
CXC chemokines			
CXCL1	GRO-α/MGSA-α	CXCR1 ,CXCR2	Neutrophils
CXCL2	GRO-β, MGSA-β	CXCR2	Neutrophils
CXCL3	GRO-γ/MGSA-γ	CXCR2	Neutrophils
CXCL4	PF4	unknown	Fibroblasts
CXCL5	ENA-78	CXCR2	Neutrophils
CXCL6	GCP-2	CXCR1, CXCR2	Neutrophils
CXCL7	NAP-2	CXCR2	Neutrophils
CXCL8	IL-8	CXCR1, CXCR2	Neutrophils
CXCL9	Mig	CXCR3	Activated T cells, natural killer cells
CXCL10	IP-10	CXCR3	Activated T cells, natural killer cells
CXCL11	I-TAC	CXCR3	Activated T cells, natural killer cells
CXCL12	SDF-1α/β	CXCR4	Resting T cells, dendritic cells, monocytes, B cells
CXCL13	BLC/BCA-1	CXCR5	B cells
CXCL14	BRAK/Bolekine	unknown	unknown
CXCL15	unknown	unknown	unknown

Table 1. Nomenclature of chemokines (adapted from Zlotnik et al, 2000) (cont.)

New systematic name of chemokine	Former name of chemokine	Chemokine receptor(s)	Cells chemoattracted or activated by chemokine
CC chemokines			
CCL1	1-309	CCR8	T cells
CCL2	MCP-1/MCAF	CCR2	Dendritic cells, basophils, monocytes, activated T cells and natural killer cells
CCL3	MIP-1α/LD78α	CCR1, CCR5	Monocytes, natural killer cells, activated T cells, dendritic cells
CCL4	MIP-1β	CCR5	Monocytes, activated T cells , dendritic cells, natural killer cells
CCL5	RANTES	CCR1, CCR3, CCR5	Eosinophils, basophils, monocytes, activated T cells, dendritic cells, natural killer cells
CCL6	Unknown	unknown	unknown
CCL7	MCP-3	CCR1, CCR2, CCR3	Eosinophils, basophils, monocytes, activated T cells, natural killer cells

Table 1. Nomenclature of chemokines (adapted from Zlotnik et al., 2000) (cont.)

New systematic name of chemokine	Former name of chemokine	Chemokine receptor(s)	Cells chemoattracted or activated by chemokine
CCL8	MCP-2	CCR3	Basophils, monocytes, activated T cells, dendritic cells, natural killer cells
CCL9/CCL10	Unknown	unknown	unknown
CCL11	Eotaxin	CCR3	Eosinophils, basophils, dendritic cells
CCL12	Unknown	CCR2	unknown
CCL13	MCP-4	CCR2, CCR3	Eosinophils, basophils, monocytes, activated T cells, dendritic cells, natural killer cells
CCL14	HCC-1	CCR1	Monocytes
CCL15	HCC-2, LKN-1, MIP-1δ	CCR1, CCR3	unknown
CCL16	HCC-4, LEC	CCR1	Monocytes, lymphocytes
CCL17	TARC	CCR4	T cells
CCL18	DC-CK1,PARC, AMAC-1	unknown	Naïve T cells
CCL19	MIP-3β/ ELC/ Exodus-3	CCR7	Naïve T cells, dendritic cells

Table 1. Nomenclature of chemokines (adapted from Zlotnik et al., 2000) (cont.)

New systematic name of chemokine	**Former name of chemokine**	**Chemokine receptor(s)**	**Cells chemoattracted or activated by chemokine**
CCL20	MIP-3α/LARC/ Exodus-1	CCR6	Naïve T cells, dendritic cells
CCL21	6Ckine/SLC/ Exodus-2	CCR7, CXCR3	T cells, B cells
CCL22	MDC/STCP-1	CCR4	Dendritic cells, T cells
CCL23	MPIF-1	CCR1	Monocytes, resting T cells, neutrophils
CCL24	MPIF-2/Eotaxin-2	CCR3	Basophils
CCL25	TECK	CCR9	Macrophages, thymocytes, dendritic cells
CCL26	Eotaxin-3	CCR3	unknown
CCL27	C-TACK/ILC	CCR10	unknown
C chemokine			
XCL1	Lymphotactin/SCM-1α/ATAC	XCR1	unknown
XCL2	SCM-1β	XCR1	unknown
CX3C chemokine			
CX3CL1	Fractalkine	CX3CR1	unknown

Abbreviations: 6Ckine (CCL21) = Six-cysteine chemokine; BCA-1(CCL13) = B-cell-attracting chemokine 1; BLC(CXCL13) = B lymphocyte chemoattractant; ELC(CCL19) = EBV-induced gene1-ligand chemokine; DC-CK1(CCL18) = dendritic cell chemokine; ENA-78 (CXCL5) = 78-amino acid epithelial cell-derived neutrophil activator; ELC(CCL19) = EBI1-ligand chemokine; FLK(CX3CL1) = fractalkine; GCP-2(CXCL6) = granulocyte chemoattractant protein 2; GCP-2(CXCL6) = granulocyte chemotactic protein-2; GRO(CXCL 1,2,3 for α,β,γ) =

growth-related oncogene; HCC-1(CCL14) = haemofiltrate CC chemokine 1; I309(CCL1) = inducible 309; IL-8(CXCL8) = Interleukin-8; IP-10(CXCL10) = interferon γ-inducible protein 10; ITAC = interferon-inducible T cell α chemoattractant; LARC(CCL20) = liver and activation-regulated chemokine; LEC(CCL16) = liver-expressed chemokine; LKN-1(CCL15) = leukotactin-1; LT(XCL1) = lymphotactin; MCP(CCL2,CCL7, CCL8, CCL13 for MCP-1,2,3 and 4 respectively) = monocyte chemotactic protein; MDC(CCL22) = macrophage-derived chemokine; MGSA(CXCL1,2,3) = melanoma growth-stimulatory activity protein; MIG(CXCL9) = monokine induced by interferon-γ; MIP(CCL3 and CCL4 for α and β respectively) = macrophage inflammatory protein; MIPF-1,2(CCL23/CCL24) = myeloid inhibitory factor 1,2; NAP-2(CXCL7) = neutrophil-activating peptide 2; PARC(CCL18) = pulmonary and activation-regulated chemokine; PF-4 (CXCL4) = platelet factor-4; RANTES(CCL5) = regulated on activation normal T-cell expressed and secreted; SDF-1(CXCL12) = stromal cell-derived factor; SLC(CCL12) = secondary lymphoid tissue chemokine; TARC(CCL17) = thymus-and activation-regulated chemokine; TECK(CCL25) = thymus-expressed chemokine.

1.7.1 IL-8/CXCL8

CXCL8 was formerly known as Interleukin-8 (IL-8). The predominant form of IL-8/CXCL8 has 72 amino acids. It has 4 cysteines forming 2 intrachain disulphide bridges which link Cys-7 to Cys-34 and Cys-9 to Cys-50 (Baggiolini et al., 1992). IL-8/CXCL8 is expressed by 'immune' cells such as mononuclear phagocytes, monocytes, T lymphocytes, neutrophils as well as many types of 'non-immune' cells such as endothelial cells, fibroblasts, keratinocytes, synovial cells, chondrocytes, epithelial cells and tumour cells which secrete IL-8/CXCL8 in response to stimulation with pro-inflammatory cytokines including IL-1α, IL-1β and TNF-α (Rollins, 1997; Baggiolini et al., 1992). IL-8/CXCL8 has been shown to recruit neutrophils in several pathological conditions such as in psoriasis, rheumatoid arthritis and adult respiratory stress syndrome. 'ELR' positive 'CXC' chemokines such as IL-8/CXCL8 contributes to tumorigenesis as a result of angiogenesis by causing migration and proliferation of endothelial cells (Moore et al., 1998; Moore et

al., 1999; Strieter et al., 1995). The expression of IL-8/CXCL8 receptors on endothelial cells is controversial and some evidence indicate that IL-8/CXCL8 may have a direct role in angiogenesis (Salcedo et al., 2000).

IL-8/CXCL8 is one of the initial chemokines to be released by epithelial cells, mainly by parietal cells (Martin-Guerrero et al., 2000) when in contact with microorganisms, hence IL-8/CXCL8 was expressed by gastric epithelial cells which were infected by *H. pylori* (Kusugami et al., 1997; Suzuki et al., 1998; Yamaoka et al., 1998; Eck et al., 2000; Rieder et al., 2001). Following its release, IL-8/CXCL8 induces an acute inflammatory response in the gastric epithelial cells by attracting neutrophils (Eckmann et al., 1993; Takagi et al., 1997). Gastric epithelial cell have also been shown to express IL-8/CXCL8 when stimulated with pro-inflammatory cytokines (Sharma et al., 1995) or viable *H. pylori* (Aihara et al., 1997)

1.7.2 MIG/CXCL9 and IP-10/CXCL10

Both of these chemokines are expressed by monocytes, fibroblasts, endothelial cells and T cells. They do not have the 'ELR' structural motif in the N-terminal regions, and are angiostatic. This angiostatic property of these two chemokines renders them suitable for use in anti-tumour chemotherapy (Strieter et al., 1995). IP-10/CXCL10 was found in higher levels in non-small cell lung cancer compared to adenocarcinoma and this resulted in angiostatic effects on the non-small–cell lung cancer (Arenberg et al., 1996, Keane et al., 1998). IP-10/CXCL10 is induced by IFN-γ and TNF-α and is expressed by monocytes, endothelial cells, keratinocytes and fibroblasts. In *H. pylori* infected gastric mucosa, MIG/CXCL9 and IP-10/CXCL10 have been shown to be expressed by the endothelial cells in the small gastric blood vessels and mononuclear cells at the sites of T cell infiltration (Eck et al., 2000). The receptor for MIG/CXCL9 and IP-10/CXCL10 is found on Th1 cells, therefore these chemokines selectively recruit Th1 cells at sites of inflammation with high production of IFN-γ (Sallusto et al., 1998) and the Th1 response contributes to gastric mucosal damage. MIG/CXCL9 and IP-10/CXCL10 were also expressed by untreated HT-29 colonic epithelial cells and HT-29 cells when they were stimulated

with a combination of IL-1α, TNF-α and IFN-γ. In normal colonic biopsies, MIG/CXCL9 and IP-10/CXCL10 were also expressed (Jordan et al., 1999).

IP-10/CXCL10 is found in high levels in psoriatic plaques and areas of chronic inflammation (Zack Howard et al.,1996). It also plays an important role in delayed-type hypersensitivity reactions.

1.7.3 CXCL12/ SDF-1α

SDF-1α/CXCL12 is the only known ligand for the chemokine receptor CXCR4 and is expressed constitutively in many organs (Nagasawa et al., 1998; Mantovani, 1999). In some cell types, SDF-1α/CXCL12 secretion appears to be independent from the effects of pro-inflammatory cytokines (Bleul et al., 1996). SDF-1α/CXCL12 stimulates the migration of endothelial cells and angiogenesis by CXCR4 despite SDF-1α/CXCL12 not having the 'ELR' motif. It is an important chemokine involved in the development of an embryo as SDF-1α/CXCL12 is responsible for haematopoiesis (B lymphopoiesis) and cardiogenesis (Nagasawa et al., 1998). A deficiency of SDF-1α/CXCL12 has been shown to result in defects in the cardiac ventricular septum and red and white blood cells in embryos (Horuk, 1998). SDF-1α/CXCL12 is normally expressed by fibroblasts, therefore it is widely found in various organs such as the brain, heart, lung, kidneys, thymus, spleen and liver. Although expressed constitutively in many tissues (Bleul et al., 1996; Sozzani et al., 1999; Murdoch, 2000), it was demonstrated that SDF-1α/CXCL12 was decreased in hepatocellular carcinoma (HCC) and other gastrointestinal cancer tissues. It was also absent in pre-malignant colonic adenomas (Begumet et al., 1996). SDF-1α/CXCL12 might have an immune surveillance role as it attracts resting T lymphocytes, monocytes and intraepithelial T cells. It also stimulates pre-B cell growth and encourages migration of B cells. SDF-1α/CXCL12 is the ligand for the CXCR4/fusin chemokine receptor which is also a co-receptor for the HIV-1 virus, therefore it can inhibit the infection of the cells by the T-tropic strains of HIV (Zlotnik et al. 1999). SDF-1α/CXCL12 is expressed on mucosal tissues which are highly relevant for the transmission and propagation of HIV (Agace et al., 2000).

The involvement of CXCR4 in HIV-1 infection meant that therapy could be aimed at blocking CXCR4 using peptides or other agents or at regulating the expression of CXCR4 or SDF-1α/CXCL12 (Nagasawa et al., 1999).

1.7.4 BCA-1/ BLC/CXCL13

This chemokine was formerly known as B-cell attracting chemokine (BCA-1) and BLC (B lymphocyte chemoattractant) and is mostly found in the liver, spleen, Peyer's Patches, mesenteric lymph nodes followed by lower levels in the lymph node, appendix, stomach, salivary gland and mammary gland (Barella et al., 1995; Legler et al., 1998). Possible sources of BCA-1/CXCL13 are dendritic cells and B lymphocytes. BCA-1/CXCL13 is highly expressed in primary and secondary lymphoid follicles and MALT lymphomas. BCA-1/CXCL13 attracts mature B cells that express its receptor, CXCR5 (Legler et al., 1998; Schaerli et al., 2000). Not many studies have been conducted on BCA-1/CXCL13, but BCA-1/CXCL13 is thought to act constitutively as a homing chemokine directing leukocyte trafficking in normal lymphoid tissue, however it is also induced by chronic *H. pylori* gastritis and in mucosal-associated lymphoid tissue (MALT) lymphomas in the stomach (Mazzucchelli et al., 1999). Therefore, BCA-1/CXCL13 has both a homeostatic and an inflammatory role.

1.7.5 MCP-1/CCL2

MCP-1/CCL2 (monocyte-chemotactic protein) is predominantly expressed by monocytes, vascular endothelial cells, smooth muscle cells, glomerular mesangial cells (Rovin et al., 1992), osteoblastic cells (Williams et al., 1992), fibroblasts, pulmonary epithelial cells (Standiford et al., 1991) and T cells. It is found in a variety of diseases such as granulomatous diseases, rheumatoid arthritis, inflammatory heart disease of the smooth muscle, endothelial and myocardial tissue, asthma, bone trauma and host responses to bacteria. MCP-1/CCL2 attracts monocytes in atherosclerosis which forms the basis of atherosclerotic plaques (Hayes et al., 1997). MCP-1/CCL2 in synergy with lipopolysaccharide may confer anti-tumour effects to

melanoma cells (Singh et al.,1993) but MCP-1/CCL2 on its own does not have any anti-tumour effects (Zack Howard et al., 1996).

MCP-1/CCL2 mRNA was expressed in unstimulated HT-29, colonic epithelial cells as well as in HT-29 cells stimulated with a combination of IL-1α, TNF-α and IFN-γ. MCP-1/CCL2 mRNA was also found in normal colonic biopsies (Jordan et al., 1999). In some gastrointestinal disease states such as Crohn's disease and ulcerative colitis, MCP-1/CCL2 has been shown to be increased and is thought to be responsible for inducing macrophages and eosinophils to the site of disease (Van Coillie et al., 1999).

1.7.6 MIP-1α/CCL3

MIP-1α/CCL3 mRNA was found in patients with *H. pylori* gastritis and there was positive correlation between the MIP-1α/CCL3 levels and the influx of mononuclear cells (Yamaoka et al., 1998). There was hardly any expression of MIP-1α/CCL3 in normal tissues. In contrast, HT-29 colonic epithelial cell line expressed constitutive MIP-1α/CCL3 (Jordan et al., 1999). Patients with duodenal ulcer (DU) expressed higher levels of MIP-1α/CCL3 in the antrum compared to the gastric body and vice versa for patients with gastric ulcer. Eradication of *H. pylori* decreased the levels of MIP-1α/CCL3 to normal levels (Yamaoka et al., 1998; Sato et al., 1999), hence MIP-1α/CCL3 may be induced by *H. pylori* infection.

1.7.7 RANTES/CCL5

Most of RANTES/CCL5 is induced by IL-1α or TNF-α, RANTES/CCL5 is produced by T cells and it stimulates the chemotaxis of T cells, eosinophils, basophils, and mast cells. Histamine in allergic reactions can be induced by RANTES/CCL5, MCP-3/CCL7 and MCP-2/CCL8, although RANTES/CCL5 is expressed earlier.

RANTES/CCL5 is often associated with diseases such as asthma, allergic rhinitis (Alam et al., 1993) and rheumatoid arthritis (Rathanaswami et al., 1993) In rheumatoid arthritis, it is expressed by human synovial fibroblasts. Interestingly, RANTES/CCL5 was also expressed in normal colonic biopsies (Jordan et al., 1999).

The role of RANTES/CCL5 in the gastric mucosa is confusing with conflicting reports. RANTES/CCL5 was present in *H. pylori*-infected stomach mucosa compared to *H. pylori* negative mucosa (Kikuchi et al., 2000). However, Yamaoka et al. (1998) found that RANTES/CCL5 mRNA but not RANTES protein was associated with *H. pylori* infection. In patients infected with *H. pylori* gastritis, immunoinflammatory cells such mononuclear cells demonstrated strong RANTES/CCL5 expression (Eck et al., 2000; Kikuchi et al., 2000). However, there was no *in vivo* expression of RANTES/CCL5 in the control tissue mucosa and in the gastric epithelial cells (Eck et al., 2000). There are no differences between the expression of RANTES/CCL5 in patients with duodenal ulcer or gastric ulcer and RANTES/CCL5 was not associated with polymorphonuclear cells. RANTES/CCL5 levels also did not decrease when *H. pylori* was eliminated (Sato et al., 1999). Therefore, more studies would have to be carried out to determine the role that RANTES/CCL5 plays in *H. pylori*-infected gastric mucosa.

1.7.8 MCP-3/CCL7

MCP-3/CCL7 is also known as monocyte-chemoattractant protein-3. It is produced by many tumour cell lines and regulates the secretion of protease by macrophages. It is important in the invasion and metastasis of cancer cells (VanDamme et al., 1992). MCP-3/CCL7 was also expressed by the HT-29 colonic epithelial cell line irrespective whether it was unstimulated or stimulated with a combination of IL-1α, TNF-α and IFN-γ. It was also found in normal colonic biopsies (Jordan et al., 1999).

1.7.9 TARC/CCL17

The former name of CCL17 was thymus and activation-regulated chemokine (TARC). TARC is constitutively expressed in the thymus at high levels therefore it may be involved in the development of the thymus. However, it can also be detected in the lungs, colon and small intestine (Imai et al., 1996) but nothing is known about its role in the stomach. TARC/CCL17 has been shown to be important in the chemotaxis of T cells (Andrew et al., 1999).

1.7.10 LARC/ MIP-3α/CCL20

LARC/CCL20 was formerly known as macrophage inflammatory protein-3α, liver and activation-regulated chemokine (LARC) and Exodus (Tanaka et al., 1999). LARC/CCL20 is expressed by the liver, thymus, lymph nodes, appendix, tonsil, lungs and peripheral blood lymphocytes (Rossi et al., 1997; Hromas et al., 1997; Power et al., 1997). It is also expressed by foetal liver and lungs (Rossi et al., 1997). LARC/CCL20 can induce chemotaxis of the peripheral blood mononuclear cells, lymphocytes and dendritic cells (Hromas et al., 1997; Power et al., 1997.). LARC/CCL20 can also inhibit the haematopoietic progenitor cell proliferation (Hromas et al., 1997) and according to Campbell et al., (1998), it can also stimulate the adhesion of a subset of memory T cells. Some LARC/CCL20 is expressed constitutively to direct the flow of leukocytes under physiological conditions, some LARC/CCL20 is expressed in neoplasia and some LARC/CCL20 is expressed in response to microbial infections, inflammatory conditions and immune signals (Mantovani, 1999).

In normal mice, murine LARC/CCL20 mRNA was expressed in the colon and small intestine and in humans LARC/CCL20 mRNA was expressed in the appendix (Tanaka et al., 1999) and the intestine (Hieshima et al., 1997). LARC/CCL20 has been shown to attract lymphocytes and dendritic cells towards the intestinal epithelial cells, therefore it may be involved in the formation and function of the mucosal lymphoid tissues.

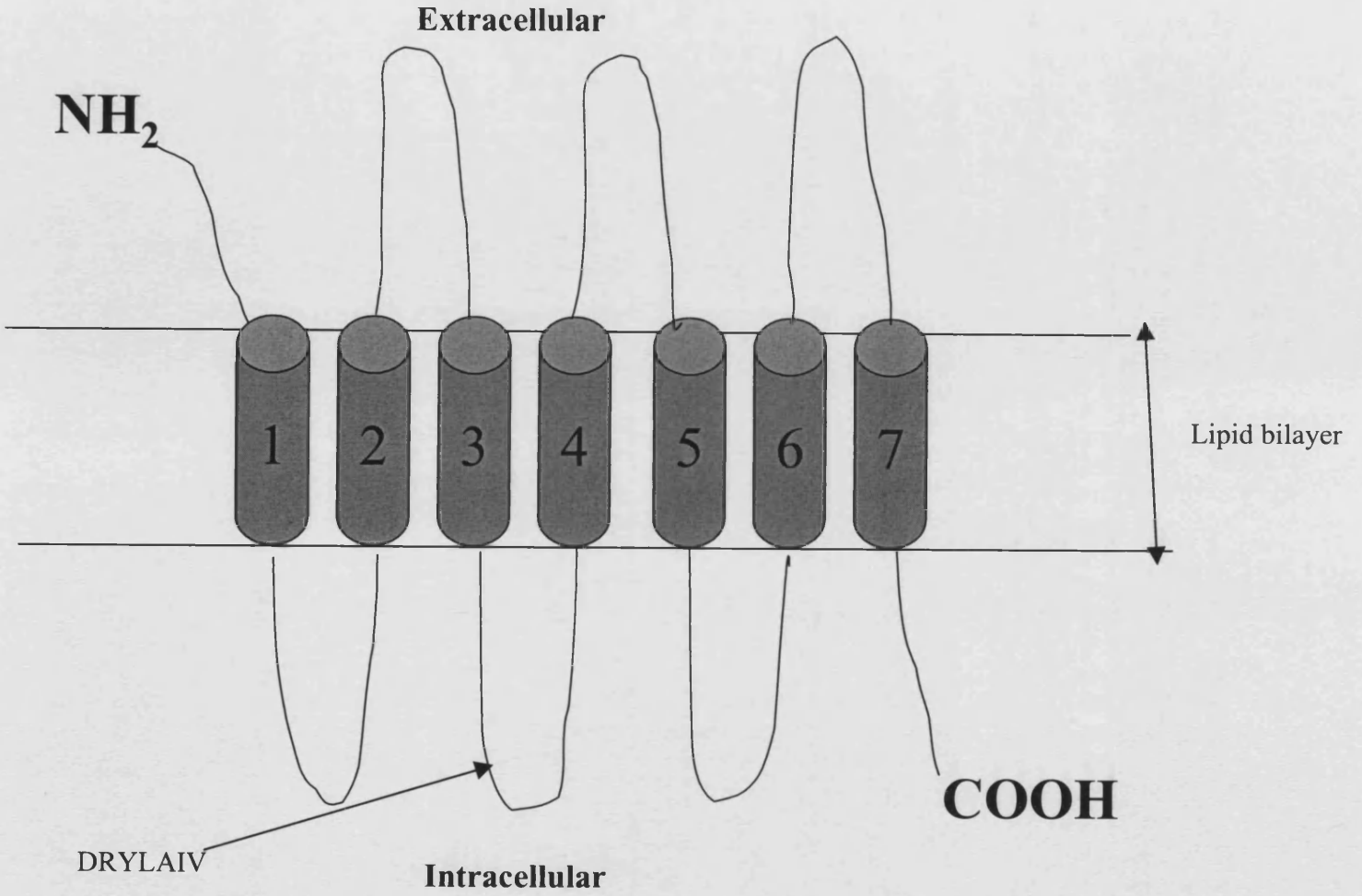
1.8 CHEMOKINE RECEPTORS

Chemokine receptors, with the exception of the Duffy antigen receptor for chemokines (DARC), are G-protein-coupled, 7-transmembrane spanning-helical proteins (Fig.6) and are found predominantly on leukocytes but are also expressed by 'non-immune' tissue cells, although less is known about this (Zack Howard et al., 1996; Rollins, 1997; Ward et al., 1998; Tachibana et al., 1998; Wells et al., 1998; Gale et al., 1999; Mahalingam et al., 1999; Rossi et al., 2000). Chemokine receptors are made up of 339-373 amino acids (Baldwin, 1993; Schertierer et al., 1993). These receptors have an extracellular N terminus tail and a cytoplasmic C terminal (Pelchen-Matthews et al., 1999). All chemokine receptors have 2 conserved cysteines situated in the NH2 terminal domain and in the third extracellular loop which form a disulfide bond important for the confirmation of the ligand-binding pocket (Baggiolini et al., 1997). Chemokine receptors have the amino acid sequence DRYLAIV in the second intracellular loop domain which makes them unique from the other 7-transmembrane receptors (Rollins,1997; Rottman, 1999). The two main sites of chemokine-chemokine receptor interaction involves the N-terminal region of the chemokine and also within the exposed loop of the chemokine backbone which extends between the second and third cysteine (Clark-Lewis et al., 1995).

The nomenclature for chemokine receptors can be classified as CXCR1-5 for the 'CXC' chemokines, CCR1-10 for the 'CC' chemokines, XCR1 for the 'C' chemokines and CX_3CR1 for the CX_3C chemokine. Most receptors are found on T cells and their expression depend on the activation state of the T cell, the activating stimuli and the growth stage. The chemokine receptors activate large GTP-binding proteins which initiates signal transduction processes via the production of second messengers such as cyclic AMP(cAMP), inositol triphosphate(IP_3), calcium ions and activated small G proteins (Goldsby et al., 2000).

Recently 'non-immune' cell types have been shown to express chemokine receptors. CXCR1, CXCR2, CXCR3 and CXCR4 were found to be expressed by endothelial cells, CCR1 and CCR2 were expressed by smooth muscle cells and the colonic epithelial cell line HT-29 expressed CXCR4 (Jordan et al, 1999; Dwinell et al.,

Fig.6 Schematic representation of a 7-transmembrane chemokine receptor



A simplified diagram of a 7-transmembrane-G-protein-coupled chemokine receptor in a cell membrane lipid bilayer. The receptor has an extracellular N-terminus marked "NH_2" and an intracellular C-terminus marked "COOH" . The hydrophobic transmembrane regions are shown as numbered cylinders embedded between the lipid bilayer.

1999). The types of chemokine receptors expressed by gastric epithelial cells were still largely unknown.

There are 4 general classes of chemokine receptors which are promiscuous receptors, shared receptors, specific receptors and virally encoded receptors (Schall et al., 1994; Rollins, 1997). Some chemokine receptors are constitutively expressed, some are inducible and some can be down-regulated. Most 'CXC' chemokines have affinities for a single receptor, whereas the 'CC' chemokines generally bind to 2 or more receptors.

Promiscuous receptors

This type of receptor will bind to both 'CXC' or 'CC' chemokines (Gale et al., 1999). Erythrocyte chemokine receptor (ECKR/DARC) is an example of a promiscuous receptor and is found on red blood cells (erythrocytes). However, the receptor has also been found on other non-erythroid cells implying it might have other regulatory roles. The tissues that express ECKR/DARC are the spleen, lungs, liver, kidneys and to endothelial cells of post-capillary venules (Horuk et al., 1993). The ECKR/DARC is thought to bind the malarial parasite *Plasmodium vivax* to red blood cells.

Shared receptors

A shared receptor will bind to more than one chemokine within the same 'CXC' or 'CC' class. Examples of shared receptors are the CXCR1 receptor which binds to IL-8/CXCL8 and any other 'CXC' chemokine with an 'ELR' protein group, CXCR3 which binds MIG/CXCL9 and IP-10/CXCL10, and also the CCR1 receptor which binds the 'CC' chemokines MIP-1α/CCL3, RANTES/CCL5, MCP-3/CCL7 and HCC-1/CCL14.

CXCR1 and CXCR2 are expressed on neutrophils, monocytes, basophils and eosinophils but all of these cells with the exception of neutrophils have a weak response to IL-8/CXCL8 (Baggiolini et al., 1994). CXCR2 has a high affinity for IL-8/CXCL8 and all 'ELR' containing 'CXC' chemokines which attract neutrophils

such as GRO-α/CXCL1, GRO-β/CXCL2, GRO-γ/CXCL3, ENA-78/CXCL5, GCP-2/CXCL6 and NAP-2/CXCL7.

CXCR3 has been found in circulating T and B lymphocytes (Qin et al., 1998) and in activated T lymphocytes and natural killer cells (Loetscher et al., 1996).

Specific receptors

This receptor only binds to one chemokine. Examples of this type of receptor are the CCR6, CXCR4 and CXCR5 receptors. CCR6 is the receptor for the chemokine LARC/CCL20 (Greaves et al., 1997). CCR6 mRNA has been shown to be expressed in the spleen, lymph nodes, thymus and appendix (Greaves, et al., 1997; Liao et al., 1999). It is also expressed in $CD34^+$ bone marrow cell-derived dendritic cells, in B cells $CD4^+$ and $CD8^+$ T cells (Power et al., 1997). CCR6, which is the receptor for LARC/CCL20 was found abundantly in normal human colonic epithelial cells (Izadpanah et al., 2001) and in other human intestinal epithelial cell lines. LARC/CCL20 may mediate autocrine and paracrine effects on the intestinal epithelium.

CXCR5 is expressed on B lymphocytes and attracts BCA-1/CXCL13 (Legler et al., 1998). This receptor is involved in the migration of B cells and memory T cells (Zlotnik, et al., 1999). The CXCR5 receptor is found mostly in the liver, spleen, lymph node, appendix, stomach, salivary gland and lower numbers of this receptor is expressed in the mammary gland (Gunn et al., 1998).

Table 2. A list of chemokines and chemokine receptors relevant to this study

CHEMOKINE RECEPTORS	LIGAND(S)
CCR1	MIP-1α/CCL3, RANTES/CCL5, MCP-1/CCL2
CCR2	MCP-1/CCL2, RANTES/CCL5, MCP-3/CCL7
CCR3	RANTES/CCL5, MCP-3/CCL7, Eotaxin/CCL11, MCP-4/CCL13
CCR4	TARC/CCL17
CCR5	MIP-1α/CCL3, RANTES/CCL5
CCR6	LARC/CCL20
CCR7	MIP-3β/CCL19
CXCR1	GRO-α/CXCL1, IL-8/CXCL8
CXCR2	GRO-β/CXCL2, IL-8/CXCL8, ENA-78/CXCL5
CXCR3	MIG/CXCL9, IP-10/CXCL10
CXCR4	SDF-1α/CXCL12
CXCR5	BCA-1/CXCL13

1.8.1 CXCR4

CXCR4 was formerly known as 'fusin' or LESTR (Loetscher et al., 1994). This chemokine receptor is expressed throughout the body, predominantly on naïve, inactive T cells (Bleul et al., 1997) and together with SDF-1α/CXCL12, CXCR4 is important in the development of the heart, blood cells as well as in the development of the neuronal networks in the central nervous system and the blood vessels in the gastrointestinal tract (Tachibana et al., 1998). CXCR4 and SDF-1α/CXCL12, its only ligand (Bleul et al., 1996; Tanabe et al., 1997) are also instrumental in the processes of embryogenesis, haematopoiesis, activation of T cells, activation of endothelial and epithelial cells, brain development and pathogenesis of HIV(Murdoch, 2000).

The formation of a mature vascular system for supplying the gastrointestinal tract is dependent on the CXCR4/SDF-1α(CXCL12) pair (Tachibana et al., 1998). This is achieved by vascular branching regulation and remodelling processes in endothelial cells. CXCR4 may also help in the apoptotic elimination of cells that have migrated incorrectly in the central nervous system (Zou et al., 1998). Mitra et al. (1999) found that the CXCR4 mRNA was expressed equally in the colon, squamous oesophageal and gastric cancers in the adjacent normal tissues. CXCR4 can be upregulated by IL-2 or phytohaematoagglutinin (PHA) stimulation of the T cells (Bleul et al., 1997) and CXCR4 can also be up-regulated by IL-4 (Jourdan, 1998; Murdoch, 2000). Conversely, IL-10 can down-regulate the expression of CXCR4 (Jinquan et al., 2000; Murdoch, 2000). When SDF-1α/CXCL12 binds to CXCR4, CXCR4 is rapidly internalised but relocates to the cell surface within minutes (Agace et al., 2000).

CXCR4 is expressed constitutively in the HT-29 colonic epithelial cell line (Jordan et al., 1999) and in inflamed and non-inflamed colonic mucosa. It has been shown by immunohistochemistry experiments that epithelial cells at the bottom of intestinal crypts or glands express CXCR4. The cells at the bottom of the glands are dividing, therefore it was believed that CXCR4 was important to stimulate cell division (Jordan et al., 1999). CXCR4 has also been shown to be expressed by endothelial cells on blood vessels. Since it is expressed constitutively by many cells, CXCR4 may have some important roles to play in cell homeostasis.

1.9 GASTRIC DISEASES (GASTRITIS)

Gastritis means inflammation of the gastric mucosa and it is the host's response to damage caused in part of the tissues. The damaged tissues release chemoattractants which attract inflammatory cells to the site of damage.

Recently, there has been great interest in gastritis because

a) it is an immunological disease associated with *H. pylori*
b) it is associated with the occurrence of gastric cancer

(Ishihara et al., 1996)

The Sydney system has been created to classify the various types of gastritis in a systematic way. According to this system, there are three categories of gastritis which are acute gastritis, chronic gastritis and gastritis of special forms (Price, 1991). *H. pylori*-associated gastritis can be characterized by increased polymorphonuclear neutrophils, chronic inflammatory mononuclear cells including macrophages and lymphocytes in the gastric mucosa (Kusugami et al., 1999).

1.9.1 Acute gastritis

Acute gastritis is known to occur in the early stages of infection with the bacterium *H. pylori*. This is characterised by an intense polymorphonuclear inflammation of the gastric antrum and body of the stomach, mainly the gastric body. The mucosal gland layer is severely damaged (epidemic achlorhydric gastritis) but heals within days or weeks (Sobala et al., 1991). Sometimes the acute gastritis can develop into chronic gastritis (Dixon, 1996).

1.9.2 Chronic gastritis

Chronic gastritis includes non-atrophic gastritis and atrophic gastritis whereby the normal gastric glands are lost, this leads to other functional consequences which vary in type and severity depending on the site of gastritis. If atrophy occurs in the antrum, there is a decrease of gastrin secretion by the G cells. Conversely, if it occurs in the body of the stomach, there will be a decrease of parietal cells (Sipponen et al., 1991). Usually atrophic gastritis can lead to intestinal metaplasia and gastric ulcers. Risk factors for acquiring atrophic gastritis are a high salt intake, intragastric bile (Sobala et al., 1993) and low dietary supply of vitamins and micronutrients (Correa, 1992). Non-atrophic gastritis usually occurs in the antrum and body, but the inflammation is mostly in the antrum (Bayerdoffer et al., 1992). Infection with the bacterium *H. pylori* is implicated in active chronic gastritis. This bacterium invokes an acute inflammatory response mediated by complement components, low-molecular weight chemotactic factors shed by the bacterium and the chemotactic cytokine IL-8/CXCL8 expressed by epithelial cells, macrophages and endothelial

cells in response to bacterial infection. Chronic gastritis is marked by infiltration of lymphocytes, plasma cells and neutrophils with lymphoid follicles and surface epithelium damage which begins in the antrum and proceeds to the gastric body (Hirai et al., 1999).

1.9.3 Special forms of gastritis

This category includes lesions which have special clinical disorders with specific pathogenesis and histological features such as eosinophilic, granulomatous or lymphocytic gastritis, reactive gastritis whereby the gastric mucosa has been subjected to noxious stimuli such as non-steroidal anti-inflammatory drugs, bile and corrosives (Sipponen et al., 1991), drugs and alcohol (Dixon, 1996). These irritants exfoliate the surface epithelial cells and decrease the secretion of mucous, causing the gastric mucosa to be prone to acid attacks. The acid attacks cause the gastric mucosa to develop vasodilation, become oedematous, form erosions and haemorrhages. If the haemorrhages are not very serious, they heal up 24-48 hours post-bleeding (Dixon, 1996).

1.10 *HELICOBACTER PYLORI (H. PYLORI)*

1.10.1 Epidemiology

H. pylori has been implicated in a number of gastrointestinal disease states such as gastric ulcers (Crowe, et al., 1995; Huang et al., 1995; Malfertheiner, 1997; Yamaoka et al., 1998; Zarilli et al., 1999; Shimizu et al., 2000), duodenal ulcers, chronic active antral gastritis (Crowe et al., 1995; Huang et al., 1995; Haeberle et al., 1997; Yamaoka et al., 1998; Zarilli et al., 1999; Martin-Guerrero et al., 2000; Lohoff et al., 2000; Shimizu et al., 2000), gastric carcinomas (Parsonnet et al., 1991; Crowe et al., 1995; Huang et al., 1995) and mucosal-associated lymphoid tissue (MALT) lymphomas (Hunt, 1996; Zarilli et al., 1999; Martin-Guerrero et al., 2000; Joo et al., 2000). Peptic/gastric ulcers are a major cause of death and distal gastric adenocarcinoma is the second biggest cancer killer worldwide (Atherton, 1997;

Vandenplas, 2000). *H. pylori* infection are very common worldwide although prevalence in the developed world is now decreasing (Tytgat et al., 2000). It mostly occurs in lower socio-economic patients as it is associated with poor hygiene, crowded living conditions, sharing of beds in childhood and poor domestic water supply (Marshall, 1994). Human beings are natural hosts of *H. pylori*. There is still uncertainty over the mode of transmission of *H. pylori* (Axon, 1996). It has been suggested that in the developed world, oral-oral contact, especially from children was the major mode of transmission, whereas in the developing world, faecal-oral was the major mode of transmission (Feldman et al., 1998).

1.10.2 The structure, microbiology and location of infection

H. pylori is a gram-negative (Yamaoka et al., 1998) spiral-shaped, microaerophillic bacillus which is 3-5 μm in length and 0.5 μm in diameter. It becomes rod-shaped when cultured in solid medium (Goodwin et al., 1990) and the bacteria converts to a coccoidal shape during unfavourable culture conditions. It has 5-6 unipolar sheathed flagella which enables it to move in the mucous layer covering the epithelial cells of the gastric mucosa (Marais et al., 1999).

H. pylori is a non-invasive bacteria and colonizes the mucous layer above the gastric epithelial cells (Bodger et al., 1998). This bacteria can only live in gastric-type epithelium and is not found in any other part of the gastrointestinal tract which does not have gastric mucosa (Harris et al., 1996).

The location of the *H. pylori* infection within the stomach determines the clinical manifestation of the disease. Generally, after ingestion, the bacterium colonises predominantly the gastric antrum (Hunt, 1996). In individuals who have low acid secretion, the bacteria will colonise both the gastric antrum and body. This leads to gastric ulcer and gastric body mucosal atrophy with increased risk of gastric cancer. In individuals with increased production of acid in the stomach, this can lead to gastric metaplasia in the duodenal bulb and there will be a greater risk of *H. pylori* colonising the duodenum which results in duodenal ulcers (Harris et al., 1996).

1.10.3 Physical adaptations

H. pylori bacteria produce the enzyme urease which aids it to survive the acidic conditions in the stomach because it does not tolerate strong acids. However, it can withstand mildly acidic conditions and when urea is present, it can tolerate pH as low as 1.5 (Hirai et al., 1999). The urease enzyme hydrolyses urea into alkaline ammonia which increases the pH of the microenvironment where the bacteria live. However, although the urease on the surface of *H. pylori* plays a very important role in the bacterial colonization of the stomach, it can also damage the epithelial cells. This is because urease attracts human leukocytes which recruits monocytes and neutrophils to induce gastric mucosal injury (Craig et al., 1992; Harris et al., 1996). The ammonia combines with water to yield ammonium hydroxide which is also directly toxic to the gastric mucosal cells and results in the formation of gastric ulcers (Smoot et al., 1990). The high concentration of ammonia also increases the permeability of hydrogen ions leading to gastric ulcers (Desai et al., 1993).

1.10.4 Virulence factors

i) Vacuolating cytotoxin (VacA)

Approximately 50% *H. pylori* strains secrete the vacuolating cytotoxin (VacA) which is an 87 kD protein that induces vacuolation and cell damage in epithelial cells in the stomach. It is only present in *H. pylori* strains which have a pathogenicity island (Axon, 1999). There are three signal sequence types, s1a, s1b and s2, which are situated at the 5' end of the gene with two mid-region types m1 and m2 in the VacA allele. The toxicity of the bacteria strain depends on the final structure of the VacA allele. The s1a/m1 strain is more toxigenic than the s1b/m1 allele (Atherton, 1998; Axon, 1999), In fact, the strains with the s1a/m1 gene are the most toxic (Shimoyama et al., 1998; Vandenplas, 2000). Possible mechanisms by which VacA can be toxic to cells are interference with the cell protective immunity (Wilson et al., 1999), affects with the antigen processing and presentation mediated by the newly made major histocompatibility complex (MHC) class II (Molinari et al., 1998) and inhibits the epidermal-growth factor mediated cell repair which leads to mucosal injury (Wilson

et al., 1999). One of the mechanisms by which expression of VacA gene increases the virulence of the bacteria is by inducing the secretion of the chemokine IL-8/CXCL8 from the gastric epithelial cells. The increase in IL-8/CXCL8 secretion can also stimulate the release of gastrin from antral G cells (Harris et al., 1996) which in turn results in increased levels of gastric acid production and the formation of ulcers.

ii) The pathogenicity island (PAI) and cytotoxin associated antigen (CagA)

This pathogenicity island (PAI) contains about 40 genes and is thought to be important in the transfer of macromolecules in the host-bacteria interaction (Axon, 1999). $CagA^+$ is one of the genes in the PAI which encodes for the 120-140kD CagA protein (Tummura et al., 1993). It consists of the *pic*A and *pic*B genomic regions (Martin-Guerrero et al., 2000). $CagA^+$ strains of *H. pylori* is associated with more severe gastritis, increased occurrence and intensity of atrophy of the antrum, peptic ulcer, intestinal metaplasia (Wilson et al., 1999), duodenal ulceration and gastric cancer (Audibert et al., 2001). The presence of the bacterial CagPAI is associated with the induction of IL-8/CXCL8 and other 'CXC' chemokines secretion which is responsible for the inflammatory processes (Audibert et al., 2001; Crabtree, 1998). This phenomenon is seen in gastric epithelial cell lines (Shimada et al., 1998). IL-8/CXCL8 attracts neutrophils to the site of infection and also acts as a pro-inflammatory cytokine which attracts other leukocytes (Hirai et al., 1999). This increases the inflammation that occurs on site. Interestingly, CagA protein is not involved directly with the IL-8/CXCL8 response (Shimada et al., 1998), instead, many genes in the CagPAI are needed to induce the IL-8/CXCL8 response (Censini et al., 1996). However, $CagA^+$ infections may not always lead to serious complications as it has been shown that $CagA^+$ is protective against adenocarcinoma of the oesophagus and oesophagogastric junction (Chow et al., 1998).

The *pic*B (promote the induction of cytokines B) and *pic*A gene in the PAI increase the virulence of the bacteria as these components are involved in increasing the production of IL-8/CXCL8 by gastric epithelial cells (Sharma et al., 1995; Tummuru et al., 1995; van Doorn et al., 1998). Another gene which has been identified is *'induced by contact with epithelium'* (*ice*A) of which there are two types, *ice*A1 and *ice*A2. *Ice*A1 is a marker for peptic ulcer disease.

iii) Lipopolysaccharide (LPS)

Lipopolysaccharides (LPS), also known as endotoxins, are carbohydrate components on the bacterial surface. Generally, the *H. pylori* LPS has low endotoxic and immunostimulatory activities compared to the LPS from other bacteria (Bliss et al., 1998; Kelly et al., 1998). *H. pylori* LPS has not been associated with high mitogenic and endotoxic activity in mice, but it can stimulate the expression of IL-6 in human peripheral blood mononuclear cells which can induce inflammation reactions (Ogawa et al., 1997). It has been hypothesised that having a less inflammatory LPS allows *H. pylori* to infect its host chronically (Bliss et al., 1998) and leads to gastric damage. LPS may also cause an autoimmune reaction from the host leading to gastric mucosal injury (Appelmelk et al., 1997; Bliss et al., 1998). Piotrowski et al. (1997) have shown that *H. pylori* LPS weakens the interactions between the mucosal cells and proteins of the extracellular matrix which leads to apoptosis (programmed cell death) of the gastric epithelial cells.

iv) Effects on gastric secretions

H. pylori infection increases the basal, post-prandial and GRP (gastrin releasing peptide)-stimulated serum gastrin levels. This in turn stimulates the parietal cells to secrete more gastric acid. Cytokines such as IL-2, TNF-α and IFN-γ also can induce the expression of gastrin (Lehmann et al., 1998). The levels of somatostatin, which inhibits acid secretion, is decreased during *H. pylori* infection, hence acid levels increase. In patients suffering from *H. pylori*-induced duodenal ulcers, histamine synthesis was found to be reduced, leading to low antral histamine content. This in turn decreases somatostatin in the antral D cells and leads to an increase in gastrin production (Harris et al., 1996).

1.10.5 Pathological effects

It is widely believed that *H. pylori* causes gastric mucosal inflammation by several possible mechanisms including the release of the IL-8/CXCL8 chemokine by the gastric epithelial cells when they come into contact with the bacteria. The release of

chemotaxic substances by the bacteria such as urease also attracts inflammatory cells (Smoot et al., 1997; Kandel et al., 2000).

The incidence of adenocarcinoma of the oesophagus and upper stomach as well as Barratt's oesophagus have also been observed to be inversely proportional to the prevalence of *H. pylori*. This was because infection with the bacteria resulted in a decrease of acid secretion. Eradication of CagPAI-positive *H. pylori* infection of the gastric body increased the acid secretion resulting in a worsening of the severity of gastro-oesophageal reflux disease and its' complications.

$CagA^{+}$VacAs1a strain of *H. pylori* attracts macrophages and T cells during chronic inflammatory reactions which releases proinflammatory cytokine migration inhibitory factor. This factor inactivates the transcription of p53 tumour suppressor resulting in formation of cancers (Hudson et al., 1999; Ibraghimov et al., 2000).

H. pylori has also been suggested to be involved in non-gastrointestinal diseases such as ischaemic heart disease, acute myocardial infarction, angina, hypertension or electrocardiographic abnormalities, although the evidence for these claims is not conclusive (Strachan, 1998). The suggested mechanisms for these coronary events are due to the induction of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 in the *H. pylori* stimulated gastric mucosa. However, many factors play a role in causing inflammation apart from the virulence of the infecting *H. pylori,* these include the host response to the infection and environmental factors (Takagi et al., 1997).

1.11 *H. PYLORI* AND CYTOKINES

Infection with *H. pylori* leads to inflammation either due to the direct injury as a result of bacterial adhesion to the cells or to the subsequent release of vacuolating cytotoxin, phospholipases, urease, cytokines and chemokines. Yamaoka et al. (1995 & 1996) found that *H. pylori* infection induced the secretion of various cytokines such as IL-6, IL-7, IL-8, IL-10 and TNF-α which contributed to the gastric inflammation associated with the infection. Gastric epithelial cells may be the cells

expressing IL-8/CXCL8 (Crabtree et al., 1994; Crowe et al., 1995) but it is not known which cells express the other cytokines although activated macrophages may secrete IL-1β and TNF-α (Sartor, 1994) and T lymphocytes or natural killer cells may secrete IFN-γ and IL-2 (Karttunen et al., 1995). Activated T lymphocytes with Th1 type characteristics have been implicated in *H. pylori* infections (D'Elios et al., 1997; Crabtree, 1998; Zarilli et al., 1999). Th1 cells boost cell-mediated immunity to cancers and intracellular infections, therefore it is paradoxical that it should be implicated in responses to *H. pylori* infection which is an extracellular/non-invasive type (Ernst et al., 1997). The Th1 response to the *H. pylori* infection is not necessarily sufficient to clear the infection, despite inducing inflammatory reactions. Mucosal and systemic antibody responses towards the bacterial antigens are also not sufficient to eradicate the infection (Blanchard et al., 1999). However, Th1 reactions could damage the gastric mucosa directly by inducing the epithelial cells to express immunogenic peptides or epitopes that could be recognised by antibodies and the overwhelming Th1 response. Additional factors including host genetics and environmental factors and the strain of the bacteria in combination could lead to epithelial damage and eventually ulcerogenesis (Ernst et al. 1997). Additionally, Th1 reactions could also cause damage indirectly when bacteria are phagocytosed.

Th1 pro-inflammatory cytokines IL-1β, TNF (Crowe et al., 1995; Yamaoka et al., 1996; Katagiri et al., 1997; Martin-Guerrero et al., 2000), IL-8/CXCL8 (Noach et al., 1994; Crowe et al., 1995; Huang et al., 1995; Yamaoka et al., 1996; Sharma et al., 1995; Ando et al., 1996; Katagiri et al., 1997; Bliss et al., 1998, Martin-Guerrero et al., 2000) and IL-6 (Crowe et al., 1995; Huang et al., 1995, Yamaoka et al., 1996; Martin-Guerrero et al., 2000) induce the expression of neutrophil or macrophage-attracting chemokines which in turn release cytotoxic material to eliminate the bacteria (Ernst et al., 1997) which also damage the host. Hida et al. (1999) extended this study and have shown that IL-10 and IL-12 mRNA were both increased in the gastric antrum and gastric body biopsies of patients infected with the $CagA^+$ *H. pylori*. IL-12 was thought to polarise the T cells into active Th1 cells (O'Garra et al., 1995; Scott et al., 1995; Crabtree, 1998) and promote cell mediated responses and is known to cause gastritis (Karttunen et al., 1997) whilst the anti-inflammatory

cytokine IL-10 down-regulated the proinflammatory responses to protect the cells against excessive 'CXC' and Th1 responses.

1.12 CHEMOKINE PRODUCTION IN RESPONSE TO *H. PYLORI* INFECTION

Investigations have shown that there appears to be a link between *H. pylori* infection and chemokine expression, mainly GRO-α/CXCL1 and IL-8/CXCL8 (Gionchetti et al., 1994; Noach et al., 1994; Moss et al., 1994; Fan et al., 1995; Peek et al., 1995; Yamaoka et al., 1995; Yamaoka et al., 1996; Ando et al., 1996). So far, only a few 'CXC' and 'CC' chemokines had been studied. There is longstanding evidence of non-immune cell production of chemokines and it has been shown that certain chemokines are expressed constitutively by the epithelial cells in the gastric mucosa, such as IL-8/CXCL8 (Crabtree et al., 1994) and SDF-1α/CXCL12 in the colonic epithelial cells (Jordan et al., 1999), which may have homeostatic roles to play. Chemokine expression may also be induced in normal gastric cells as a result of exposure to various stimuli such as dietary components (Yamaoka et al., 1998). Most of the studies to date have been carried out to look for IL-8/CXCL8 and there is less information available regarding the newer chemokines.

Infection of the gastric epithelial cell lines and gastric mucosa by *H. pylori* induces the production of chemokines. Neutrophils are recruited to the site of inflammation by IL-1 and TNFα, the pro-inflammatory cytokines which are released from macrophages as a result of the infection. Neutrophils release chemokines which amplify the inflammatory response to the infection (Crabtree, 1997). 'CXC' chemokines play a more important role than 'CC' chemokines in recruiting the neutrophils and T lymphocytes to the sites of inflammation (Shimoyama et al., 1998; Yamaoka et al., 1998).

KATO III and AGS gastric epithelial cell lines have been shown to express IL-8/CXCL8 mRNA constitutively (Sharma et al., 1995). IL-8/CXCL8 has an important role to recruit and activate inflammatory immune cells and gastric epithelial cells

(Eck et al., 2000). KATO III and MKN45 cell lines also expressed IL-8/CXCL8 in response to pro-inflammatory cytokines such as IL-1α and TNF-α (Yasumoto et al., 1992).

It has been shown that different strains of *H. pylori* induce different quantities of IL-8/CXCL8 secretion by epithelial cells, $CagA^+$ *H. pylori* induce more IL-8/CXCL8 than the $CagA^-$ strain (Crabtree et al., 1994; Ernst et al., 1997). Stimulation of the KATO III and MKN45 gastric epithelial cell lines were more successful in producing IL-8/CXCL8 when live *H. pylori* was used rather than killed *H. pylori*, cell-free supernatants from *H. pylori* cultures or bacterial lipopolysaccharides (Aihara et al., 1997). Similarly, stimulation with sonicated or freeze-thawed viable *H. pylori* resulted in the expression of GRO-α/CXCL1, IL-8/CXCL8, MCAF/CCL2, MIP-1α/CCL3 and MIP-1β/CCL4 by KATO III and MKN45 cells (Yamaoka et al., 1997). When the AGS gastric epithelial cell line was stimulated with viable *H. pylori*, IL-8/CXCL8 mRNA peaked early post-stimulation, but ENA-78/CXCL5 peaked later around 90 minutes post-stimulation (Rieder et al., 2001). Due to its delayed and long-lasting effects, ENA-78/CXCL5 could be involved in the recruitment of granulocytes in acute and chronic infections. Other chemokines released by KATO III cells such as GRO-α/CXCL1, IL-8/CXCL8, MCP-1/CCL2 and MIP-1α/CCL3 decreased when *H. pylori* was eradicated. KATO III cells expressed intercellular adhesion molecule-1 (ICAM-1), the adhesion molecule for leukocytes when these cells were stimulated with pro-inflammatory cytokines such as IL-1, TNF-α and IFN-γ (Sharma et al., 1995). However, stimulation of the KATO III cells with *H. pylori* did not result in the expression of ICAM-1.

In a study performed upon antral biopsy cultures from patients with and without *H. pylori* infection, the chemokines GRO-α/CXCL1 (Kusugami et al., 1997; Suzuki et al., 1998), IL-8/CXCL8 (Kusugami et al. 1997; Ando et al., 1998; Suzuki et al., 1998; Rieder et al., 2001), MIP-1α/CCL3 (Ando et al., 1998) and ENA-78/CXCL5 (Rieder et al., 2001) were elevated in *H. pylori* positive patients. In *H. pylori*-infected gastric mucosa, macrophages expressed mainly GRO-α/CXCL1 (Ando et al., 1996; Kusugami et al., 1997), IL-8/CXCL8 (Ando et al., 1996; Kusugami et al., 1997) and MIP-1α/CCL3 (Suzuki et al., 1999).

The expression of four chemokines mainly GRO-α/CXCL1, IL-8/CXCL8, MCP-1/CCL2 and MIP-1α/CCL3 were studied in patients with *H. pylori*-associated gastric ulcers and *H. pylori*-associated duodenal ulcers (Sato et al., 1999). As expected, GRO-α/CXCL1 and IL-8/CXCL8 expression were associated with increased myeloperoxidase which is synonymous with the numbers of neutrophils.

Antral biopsies from patients with duodenal ulcers and gastric ulcers are also known to express higher levels of GRO-α/CXCL1 and IL-8/CXCL8 compared to patients with gastritis or normal patients (Ohsuga et al., 2000). The expression of IL-8/CXCL8 and MIP-1α/CCL3 in the antrum and at the gastric ulcer sites of patients with and without *H. pylori* infection was studied. As expected, there were higher levels of expressed IL-8/CXCL8, MIP-1α/CCL3 and inflammatory cells in the antrum and ulcer sites of patients positive for *H. pylori* infection compared to patients negative for *H. pylori*. However, the ulcer sites expressed more IL-8/CXCL8, MIP-1α/CCL3 and inflammatory exudate than the antrum (Shimizu et al., 2000).

2.0 AIMS OF THE STUDY

The bacteria *Helicobacter pylori* have been implicated in various diseases in the stomach which can lead to complications such as gastric ulcers, particularly duodenal ulcers, gastric cancers and mucosa-associated lymphoid tissue lymphomas. This study, has focused on gastritis, as this is a precursor of various complications.

It is now known that some chemokines, especially IL-8/CXCL8, which are proteins involved in attracting leukocytes to the site of infection are responsible for the inflammation associated with the *H. pylori* infection.

This study aims to investigate the role of gastric epithelial cells and their chemokine production in the initiation and propagation of inflammatory events in the gastric mucosa and the role of chemokines and receptors in normal gastric epithelial cell function.

1) Gastric epithelial cell lines have been used as a model to investigate the expression of members of the chemokine families, especially recently identified chemokines like BCA-1/CXCL13 and LARC/CCL20.

2) To investigate the regulation of IL-8/CXCL8 and RANTES/CCL5 chemokine expression by the cytokines IL-4 and IL-13.

3) To determine using RT-PCR and immunohistochemistry whether the chemokine expression in gastric biopsies is associated with inflammation or *H. pylori* infection, with an emphasis on recently identified chemokines.

4) To determine by RT-PCR which chemokine receptors are expressed by gastric epithelial cells

Although the aetiology of gastric diseases as a result of *H. pylori* infection is still not fully understood, it is hoped that we might gain some useful insight as to the possible mechanisms involved in causing gastric damage. Increasing our understanding of chemokine production by gastric epithelial cells may provide leads for the

development of novel therapeutic strategies targetting chemokines or chemokine receptors to decrease inflammation associated with *H. pylori* infection.

3.0 MATERIALS AND METHODS

METHODS

3.1 CELL CULTURE

3.1.1 MKN45 and AGS gastric epithelial cell lines

Two human gastric epithelial cell lines were used in these studies, MKN45 and AGS. The MKN45 cells were obtained from the Japanese Collection of Research Bioresources (JCRB) and had been isolated from a poorly differentiated adenocarcinoma in the stomach of a 62 year old female. The MKN45 cells grow in a monolayer with clusters of cells containing oval and spindle shaped cells.

The AGS cells were obtained from the European Collection of Animal Cell Cultures (ECACC) and had been isolated from a stomach adenocarcinoma in a 54 year old Caucasian female. Both cell lines have been frequently used as models of the gastric epithelial mucosa in other previous studies of AGS (Rieder et al., 2001) and MKN45 (Mori et al. , 2001; Ohsuga et al., 2000).

Two other human cell lines, HT-29 and U937 were used as positive controls for the reverse-transcriptase polymerase chain reaction (RT-PCR) experiments (section 2.6). The HT-29 and U937 cell lines were used by colleagues within our laboratory, and had been obtained from the European Collection of Animal Cell Culture (ECACC). The HT-29 cells had been isolated from a colorectal adenocarcinoma in a 78 year old female and had an epithelial cell morphology. The HT-29 cells grow in an adherent monolayer. The U937 cell line is a myeloid leukaemia cell line obtained from a 37 year old Caucasian male. These cells grow in suspension.

3.1.2 Cell culture

MKN45 cells were cultured in 80cm^2 tissue culture flasks in RPMI 1640 medium containing 2mM glutamine supplemented with penicillin (10U/ml), streptomycin (10μg/ml), amphotericin B/Fungizone® (0.5μg/ml) and 10% heat-inactivated foetal bovine serum (referred to as a complete medium). The U937 cells were cultured in the same conditions.

AGS cells were cultured in 80cm^2 tissue culture flasks in Ham's F12 Nutrient medium containing 2mM glutamine supplemented with penicillin (10U/ml), streptomycin (10μg/ml), amphotericin B/Fungizone® (0.5μg/ml) and 10% foetal bovine serum (referred to as a complete medium).

HT-29 cells were cultured in 80cm^2 tissue culture flasks in McCoy's 5A medium containing 2mM glutamine supplemented with penicillin (10U/ml), streptomycin (10μg/ml), amphotericin B/Fungizone® (0.5μg/ml) and 10% foetal bovine serum (referred to as complete medium).

Cultures of the MKN45, AGS, HT-29 and U937 were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. The medium was changed every 2-3 days.

To subculture the cells, the medium was removed and the cells were washed twice with phosphate buffered saline solution (PBS) without calcium and magnesium. The excess solution was removed and 3 ml of trypsin/EDTA mixture containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA were added. The cells were incubated for 5 minutes at 37°C until they had detached from the flask. Ten ml of complete medium were added to the cell mixture to inhibit the reaction of the trypsin/EDTA solution. Cells were centrifuged at 200g for 5 minutes. After centrifugation, the supernatant was removed and the cell pellet was resuspended in complete medium. A count of viable cells was carried out by mixing a small volume of cells with an equal volume of 0.02% trypan blue in PBS. The cells were counted using a Neubauer haemocytometer. Dead cells absorbed trypan blue into the cells and stained blue.

Cell viability was generally more than 95%. Cells were counted and seeded for further culture at $2x10^4$cells/ml of medium into 80cm^2 tissue culture flasks. For experimental protocols, cells were seeded at the same density into 24 well plates or 25cm^2 tissue culture flasks. AGS, MKN45 and HT-29 cells reached confluency after approximately 7 days when 1ml of resuspended AGS or MKN45 or HT-29 cells were seeded together with 10ml of complete medium in a 80cm^2 flask. The U937 cell line reached the appropriate density to be harvested after approximately 5 days in culture.

For storage, cells were resuspended in $4x10^6$ cells/ml of freeze medium which contained 10% dimethylsulphoxide (DMSO) and 90% foetal bovine serum. The cell suspension was transferred to cryovials (Nunc) at 1 ml/tube which were cooled gradually overnight in the vapour phase of liquid nitrogen and then stored in liquid nitrogen. When the cells were required again, the cryovials were rapidly defrosted at 37°C in a water bath, washed in the appropriate tissue culture medium and cells from 1 cryovial were resuspended in complete medium and plated out as usual.

3.1.3 Experimental protocol

MKN45 cells were grown in 25cm^2 flasks or 24 well plates until confluent, while AGS cells were grown until confluent in 80cm^2 flasks or 24 well plates until confluent, depending on the experiments to be carried out. MKN45 and AGS cells were seeded into 24 well plates for ELISA experiments and in 25cm^2 and 80cm^2 flasks for Northern analysis and reverse-transcriptase polymerase chain reactions (RT-PCR) experiments respectively. The HT-29 and U937 cell lines were seeded into 80cm^2 flasks for RT-PCR experiments. The freshly isolated human white blood cells (refer to section 3.4.1) were seeded into small 5ml petri dishes and incubated with a combination of medium and phytohaemagglutinin (PHA) or medium and lipopolysaccharide(LPS) for a short period (2-4 hours) to be used in the RT-PCR experiments.

Twenty four hours before an experiment, these cells were usually 'serum starved', that is were cultured in their respective media without foetal bovine serum. The

absence of serum ensured that all cells were growth-arrested. These growth-arrested cells were then treated with fresh serum-free medium and treated with the specific doses of cytokines or vehicle controls for the stated times described in the results section. For ELISAs, the supernatants were collected, centrifuged to remove cellular debris and stored at -70°C until assayed for extracellular chemokines. Total RNA and cellular proteins were extracted from the cells as described in the section below.

3.2 NORTHERN ANALYSIS

Total cellular RNA was extracted from MKN45, AGS cells or biopsy samples using a commercial solution containing guanidinium thiocyanate (RNAzol B®), with a modification of the method described by Chomszynski and Sacchi (1987). Buffers and solutions used for Northern analysis are detailed in section 3.8.2 and all solvents were high performance liquid choromatography (HPLC) grade.

3.2.1 RNA isolation

Monolayers of MKN45 cells in 25cm^2 tissue culture flasks and AGS cells in 80cm^2 tissue culture flasks were lysed by adding 1 ml of RNAzol B® per 80 cm^2 flask. The cells were removed with a sterile cell scraper and transferred to sterile 1.5ml centrifuge tubes by plastic pipettes. To extract total RNA, 100µl of chloroform was added to each 1ml of homogenate, vortexed and kept on ice for 5 minutes. Then the homogenates were centrifuged at 12000g for 15 minutes at 4°C. Using a sterile pipette, 0.5ml of the upper, clear layer was transferred to a fresh sterile 1.5ml centrifuge tube and 0.5ml of propan-2-ol was added to it. The mixture was left on ice for 15 minutes before it was centrifuged again at 12000g for 15 minutes. After centrifugation, the supernatant was discarded and the white RNA pellet at the bottom of the tube was washed with 1ml 75% cold ethanol and centrifuged again at 12000g rpm for 8 minutes at 4°C. The contents of the centrifuge tube was frozen at -70°C. The sample was used directly for Northern analysis or purified further for PCR.

3.2.2 RNA measurement and sample preparation

After overnight storage at -70°C, the 75% ethanol was discarded, the pellet was rewashed in 1 ml of fresh 75% cold ethanol, vortexed and centrifuged at 12000 g for 8 minutes. Then the ethanol was removed and the RNA pellet was air dried in a fume cupboard for an hour. When the pellet was dry, it was solubilised in 20-40 µl of DEPC-treated water and kept on ice.

Total RNA was measured using a Gene Quant spectrophotometer. Two µl of RNA was diluted into 100 µl of DEPC-treated water in a cuvette and its absorbance was read at 260nm. Optical density readings were also obtained at 230nm or 280nm to assess the purity of the RNA pellet. Low protein contamination was shown by a ratio of less than 1.7 for the OD:260nm:OD280nm and a low OD230nm:OD260nm ratio indicated low guanidine contamination.

For Northern analysis, 30µl of sample buffer containing ethidium bromide, was added to 5-7 µg of sample and the samples were vortexed mixed before they were heated for 15-30 minutes at 80°C in a heating block. The samples were then cooled on ice and stained with 2.5 µl bromophenol blue solution. The samples were vortex mixed and briefly centrifuged before they were loaded onto 1% agarose gels.

3.2.3 Gel preparation and transblotting

One percent agarose gel was prepared by dissolving 3.6g of agarose (Roche) into 280ml sterile MilliQ water. Eighteen ml of 20xMOPS and 65 ml of formaldehyde were added to the dissolved agarose and the gel was cooled to about 60°C before pouring. The gel was set in a 15x25 cm gel tray with two 15 lane combs purchased from Bio-Rad.

After the gel had set in the gel tank, it was transferred to a Bio-Rad submarine tank which was filled with cold 1xMOPS running buffer. RNA (5-7 µg calculated from

the spectrophotometer readings) was loaded per lane and the gel was run at a current of 100mA until the bromophenol blue had migrated about 5cm. The gel was placed on a ultra-violet transilluminator and the ethidium bromide stained 18S and 28S ribosomal RNA bands were examined for equal loading. The gel was then photographed using a Polaroid CU5 88-46 land camera (Genetic Research Instrumentation Ltd.) and type 55 Polaroid film (Sigma). The gel was rinsed gently in sterile MilliQ water for 30-60 minutes to remove the formaldehyde, prior to transblotting, as the formaldehyde would interfere with the transfer of RNA onto the nylon membrane.

The blotting tank consisted of a glass plate suspended in a plastic container which was partially filled with 20xSSC buffer (made up of sodium citrate and sodium chloride). A strip of Whatman filter paper was placed on the glass plate with its ends reaching into the buffer solution acting as a wick to draw up the 20xSSC buffer. The gel was placed upside down on the filter paper and covered with a positively charged nylon membrane and 3 pieces of filter paper cut a similar size as the nylon membrane, which had all been wet with the 20xSSC buffer. A stack of paper towels were then placed on top of the filter papers, followed by a weight of 500g on top. This was left overnight at room temperature to allow the RNA to transfer from the agarose gel onto the nylon membrane by capillary transfer. The RNA was baked in an oven at 120°C for 20 minutes to allow the RNA to be fixed onto the membrane. The nylon membrane was then sealed in a polythene bag at room temperature before it was hybridised.

3.2.4 Hybridisation with Dig-labelled oligonucleotide probes

Nylon membranes were hybridised with Digoxigenin labelled oligonucleotide probes and the probes which were bound to the mRNA were detected following the method described in the Digoxigenin(DIG) chemiluminescent detection kit for Northern blotting from Roche. A steroid hapten, digoxigenin, was used to label the nucleic acid probes, this enabled an alkaline phosphatase anti-conjugated DIG antibody to be used in the detection system. The 100cm^2 membrane was prehybridised with 20ml hybridisation solution in a bag for an hour at 42°C. The prehybridisation solution was discarded and 1ml of 10ng/ml IL-8/CXCL8 or RANTES/CCL5 RNA probe

diluted in hybridization solution was added to the membrane and the plastic bag was sealed, ensuring air bubbles were not trapped, and left at 42°C overnight. The following day, the membrane was washed twice at 42°C with 2xSSC containing 0.1% SDS for 5 minutes followed by two washes with 0.1xSSC containing 0.1% SDS for 5 minutes per wash. The membrane was then washed for 5 minutes at room temperature in washing buffer on a rocking platform followed by a 30 minute wash with buffer 2 to block any non-specific binding of probes to the membrane. The membrane was incubated for 30 minutes on a rocking platform with a Dig-labelled antibody diluted 1:10000 in buffer 2 followed by 2 washes with a wash buffer for a duration of 15 minutes per wash. The membrane was then equilibrated with buffer 3 for 5 minutes and then drained and incubated for 5 minutes in between 2 pieces of plastic sheet with 1ml of CSPD substrate diluted 1:100 in buffer 3. After the membrane was drained again, it was sealed inside a plastic bag and incubated in a dark place at 37°C for 10-15 minutes. The membrane was then exposed to a Kodak X-Omat AR5 X-ray film (Sigma) for about 2 hours at room temperature in the dark room. The film was then developed using an automatic RGII Fuji X-ray film developer.

3.2.5 Stripping membranes for β-actin analysis

The nylon membranes which were tested for IL-8/CXCL8 and RANTES/CCL5 mRNA expression were subsequently stripped of the chemokine probes in order to probe them for the housekeeping gene, β-actin. This step was important to prove that equal loading of cellular mRNA was performed for each well.

SDS(0.1%) solution was made up using sterile MilliQ water. About 100ml was prepared per 100cm^2 membrane. The SDS solution was microwaved in a glass beaker until it was boiling. Following this, the nylon membranes were taken out of their sealed polythene bags and put into the beaker containing the boiling SDS solution. The membranes were swirled around in the beaker to ensure they were evenly exposed to the SDS solution and left until the SDS solution cooled to room temperature. This was repeated 1X in freshly prepared boiling SDS. The nylon membranes were removed from the SDS solution, prehybridised in hybridisation

solution and hybridised overnight (as described in section 3.2.4) with DIG-labelled β-actin probes. The following day, these membranes were washed and developed as in section 3.2.4.

3.3 ENZYME-LINKED IMMUNOABSORBENT ASSAY (ELISA)

The sandwich ELISA technique was used to quantitate antigenic chemokine secretion into cell culture media supernatants. All ELISA samples were measured in duplicate. All the recipes for buffers and solutions can be found in section 3.8.3.

3.3.1 IL-8/CXCL8 and RANTES/CCL5 ELISA

The monoclonal capture anti IL-8/CXCL8 and RANTES/CCL5 antibodies were diluted in coating buffer to a concentration of 2μg/ml in PBS (pH 7.4) buffer. Immediately 50μl were added per well of 96 well microtitre plates and the plates were covered and left overnight at room temperature. The wells were aspirated and washed three times with wash buffer and 100μl of blocking buffer was added to each well and incubated for 1 hour at room temperature. Following this, the plates were washed three times with wash buffer and the IL-8/CXCL8 or RANTES/CCL5 standards and culture supernatants diluted in a dilution buffer were loaded into the wells in duplicates and were incubated at room temperature for 2 hours. The same standard concentration range of 0.02-2ng/ml was used for both the IL-8/CXCL8 and RANTES/CCL5 standard curves. The plates were washed three times with wash buffer and 100μl of 20ng/ml of biotinylated anti-human IL-8/CXCL8 detecting antibody or 5ng/ml of biotinylated anti-human RANTES/CCL5 detecting antibody was added onto each well and the plates were incubated at 37°C for 1 hour.

The plates were then washed three times with wash buffer and 50μl/well of streptavidin peroxidase (0.2μg/ml) was added to each well on the plates and they were incubated at 37°C for 30 minutes. The plates were washed another three times with wash buffer. Fifty ml of substrate solution was prepared, containing hydrogen peroxide and a 10mg OPD tablet. This was added to each well at a volume of 100μl.

The plates were incubated in the dark for 30 minutes for IL-8/CXCL8 and 45 minutes for RANTES/CCL5 to allow for colour development. After this time, the reaction was quenched by adding 150µl of 1M H_2SO_4 to each well. The optical density of the plates was determined at 490nm on Dynatech MR500.

3.4 REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Polymerase chain reaction is a technique which is used to amplify regions of known size in chemokine and housekeeping mRNA of interest using specific primers. All recipes for buffers and solutions are found in section 3.8.4.

3.4.1 Preparation of white blood cells as positive controls for PCR

About 20ml of blood was taken from a donor using a needle and syringe containing 50µl heparin. Blood was centrifuged in a sterile Falcon tube at 450g for 10 minutes. The plasma layer was discarded and an equal volume of RPMI medium was added to the packed cell volume at the bottom of the tube. The RPMI medium did not contain any added antibiotics, anti-fungals or foetal bovine serum.

Four ml of 6% dextran at room temperature was added to the packed cells in RPMI medium. The contents were inverted and mixed well and the mixture was left at room temperature for 30 minutes.

After 30 minutes, the contents of the Falcon tube had separated into 2 layers. The supernatant containing white blood cells was transferred to another sterile Falcon tube and washed with a large volume of RPMI medium and centrifuged at 450g for 10 minutes. After centrifugation, a pellet of cells was obtained and the supernatant was discarded. Two ml of cold sterile MilliQ water was added to the pellet of cells to lyse the erythrocytes (red blood cells) present in the cell pellet. The cells were gently mixed with the cold water and after 1 minute, RPMI medium was added to the cells which were washed by centrifugation for 10 minutes at 450g. If the pellet of cells

still appeared red in colour, the erythrocytes were lysed again using 2ml of cold sterile MilliQ water. The white blood cells obtained were used in the RT-PCR experiments as a control.

If unstimulated white blood cells were needed, 1ml of RNAzol B® was added immediately to the white blood cells, the cells were pipetted, mixed well and lysed before they were stored in the -80°C freezer until future use.

If stimulated white blood cells were needed as positive controls, the white blood cells were added to about 4ml of complete RPMI medium, and seeded into a little culture dish. It was then stimulated with either 50ng/ml leucoagglutinin (PHA-L) or 50ng/ml phorbol 13-myristate 12-acetate (PMA) for 2-4 hours.

3.4.2 Total RNA isolation

Total RNA was isolated from AGS and MKN45 cells as described in 3.2.1. The same total RNA isolation method as described in section 3.2.1 was also performed on the HT-29, U937 and white blood cells which were used as positive controls in the PCR experiments. Total RNA was also extracted from gastric mucosa biopsies using 4-5 pieces of gastric biopsies from the antrum or body of the stomach homogenised on ice with a glass homogeniser using 1ml of RNAzol B® solution.

3.4.3 Sample preparation

The total purified RNA pellets derived from AGS, MKN45, HT-29, U937, white blood cells and gastric mucosa biopsies were solubilised in 40µl sterile MilliQ water and the quantity of nucleic acid was measured by measuring the absorbance at 260nm (section 3.2.2).

3.4.4 DNAse treatment of RNA preparations

A DNAse treatment step was included to destroy any genomic DNA contaminants which might exist in the sample and give a false positive result in the reaction. The DNA is destroyed by using a DNAse I enzyme which works optimally in the presence of DNAse I reaction buffer provided in the kit. One μg of total RNA from each sample was made up to a volume of 8μl with DNAse/RNAse free water (nuclease free water). This was done in duplicate tubes enabling a negative control to be included in the PCR for each sample. One μl of 10xDNAse reaction buffer was added to each PCR tube followed by 1 μl of DNAse I enzyme. This mixture was left at room temperature for 15 minutes. One μl of 25mM EDTA was added to each tube to inactivate the DNAse reaction and the sample was heated for 10 minutes at 65°C in the Perkin Elmer Gene Amp 2400 thermocycler machine (Warrington, UK.)

3.4.5 Reverse-transcription

This step enabled the synthesis of complementary DNA (cDNA) from the RNA present in the sample, which is the template used in the PCR step. Following the DNAse treatment, 2 μl of oligo$(dT)_{12\text{-}18}$ (5μM) primer were added to each tube and the samples were denatured at 70°C for 10 minutes. The samples were then immediately cooled on ice. Eight μl of a reaction mixture was added to each sample in 0.2 ml thin wall PCR tubes which contained 4μl of 5x reverse transcription buffer (50 mM Tris HCl pH 8.3, 75 mM KCN, 3 mM $MgCl_2$), 1μl of 10 mM DTT, 1μl of dNTP (made up from a combination of 0.5mM each of dATP, dCTP, dGTP and dTTP), 1μl of 1U/μl RNAsin, a non-competitive ribonuclease inhibitor and 1μl of Superscript®.

The reverse-transcription enzyme(Superscript®) was not added to a duplicate tube of each sample (RT minus). This acts as a control for the RT positive sample indicating that it was not contaminated with genomic DNA.

The PCR tubes were placed in a Perkin Elmer Gene Amp 2400 thermocycler (Warrington, UK) and followed a reverse transcription programme of : 42°C for 60 minutes, 95°C for 5 minutes and hold at 4°C until the samples were removed. The RT products were either used immediately or stored long-term at -80°C.

3.4.6 Polymerase chain reaction (PCR)

The NCBI Genebank programme which is available on the internet was searched to identify human chemokine and chemokine receptor mRNA or cDNA sequences. Forward and reverse primers which were each 20 nucleotides long, with an optimum G (guanine) and C (cytosine) composition and with annealing temperatures between 55°C and 65°C were designed using Primer 3 Design software on the internet using the sequences obtained from Genebank (Table 3). These primers were designed to generate product sizes between 200-500 base pairs. The specificity of primers for the required chemokine or receptor only was checked using the NCBI BLAST programme on the internet. Oligonucleotide primers were synthesised by Gibco.

Each RT template from an experimental sample was tested for expression of various CXC, CC chemokines and chemokine receptors. Each experiment included the reverse-transcribed sample, a negative control of nuclease-free water and the RT minus control which should indicate any contamination with genomic DNA. Each PCR reaction was carried out in a volume of 25µl in 0.2 ml PCR tubes consisting of 2.5 µl of PCR buffer and (1.5mM) Mg^{2+} (Roche), 200µM (final concentration) of the 4 deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP making a total of 0.5 µl dNTP mix, 15.6µl of nuclease free water, 2µl (500 nM-final concentration) of forward primer, 2µl (500 nM-final concentration) of reverse primer, 0.125µl (0.05 U/µl) of Expand High Fidelity PCR system containing the two DNA polymerase enzymes Taq and Pwo. 1.25µl of template cDNA (0.1-0.75µg) was added to the PCR tubes. The polymerase enzymes and PCR buffers were used according to 'Expand™ High Fidelity PCR system' manufacturer's specifications and the thermocycler was operated using the conditions listed below (Fig.7): 5 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at the annealing temperature between 56°C-65°C

Table 3. RT-PCR Primers

Probe	Product length (bp) & Annealing temperature (°C)	Sense primer sequence	Antisense primer sequence	Accession no.
CCR1	327 bp (56°C)	ACCTGCAGCCTTCACTTTCCTCAC	GGCGATCACCTCCGTCACTTG	L09230
CCR2	255 bp (56°C)	CCAACTCCTGCCTCCGCTCTA	CCGCCAAAATAACCGATGTGATAC	U03882
CCR3	315 bp (56°C)	TGGCGGTGTTTTTCATTTTC	CCGGCTCTGCTGTGGAT	U28694
CCR4	349 bp (57°C)	GAAGAAGAACAAGGCGGTGAAGAT	ATGGTGGACTGCGTGTAAGATGAG	X85740
CCR5	280 bp (56°C)	TGCTACTCGGGAATCATAAAAACT	TTCTGAACTTCTCCCCGA`CAAA	U54994
CCR6	266 bp (56°C)	ATCGTAATGAAGTTGGGGTT	ATCACAAATTTCAGACCCCT	U45984
CCR7	362 bp (56°C)	ACTCCATCATTTGTTTCGTG	TAGTATCCAGATGCCCACAC	L31581
CCR8	286 bp (56°C)	AGTATGCACATCTTGGATGG	TGTAGTCTACGCTGGAGGAA	U45983

Table 3. RT-PCR Primers (cont.)

Probe	Product length (bp) and Annealing temperature (°C)	Sense primer sequence	Antisense primer sequence	Accession no.
CCR9/10	579 bp (56°C)	CAAAGTCTTCCTCCCCCAGTCT	ACCAAGACACAACCAATACG	U94888
CXCR1	363 bp (58°C)	GGGGCCACACCAACCTTC	AGTGCCTGCCTCAATGTCTCC	L19591
CXCR2	385 bp (57°C)	CCGGGCGTGGTGGTGAG	TCTGCCTTTTGGGTCTTGTGAATA	M73969
CXCR3	293 bp (55°C)	CTCCACCTAGCTGTAGCAGA	AGGAAGATGAAGTCTGGGAG	X95876
CXCR4	206 bp (56°C)	TTCTACCCCAATGACTTGTG	ATGTAGTAAGGCAGCCAACA	X71635
CXCR5	465 bp (56°C)	ATCTTCTTCCTCTGCTGGTG	GTTCCTCTAGCTACCCCAAA	X68149
CXCL8/ IL-8	562 bp (65°C)	TGGGTGCAGAGGGTTGTG	CAGACTAGGGTTGCCAGATTTA	M28130
CXCL9/ MIG	214 bp (56°C)	AAGAAGCACGTGGTAAAACA	TCTCGGTGGCTATCTTGTTA	X72755

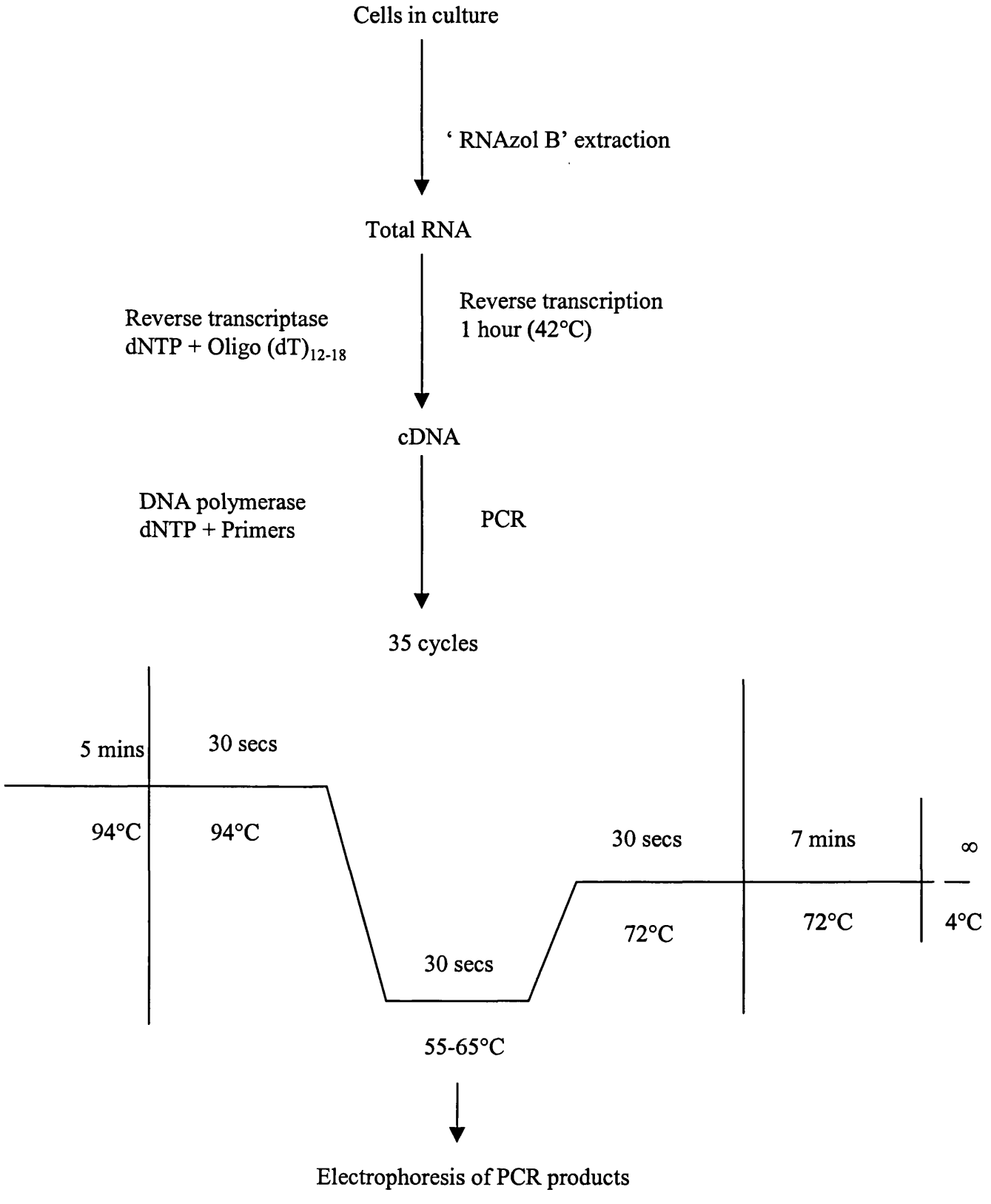
Table 3. RT-PCR Primers (cont.)

Probe	Product length (bp) and Annealing temperature (°C)	Sense primer sequence	Antisense primer sequence	Accession no.
CXCL10/ IP-10	229 bp (56°C)	CCTGCTTCAAATATTTCCCT	CCTTCCTGTATGTGTTTGGA	X02530
CXCL12/ SDF-1α	132 bp (56°C)	AGAGATGAAAGGGCAAAGAC	CGTATGCTATAAATGCAGGG	U19495
CXCL13/ BCA-1	199 bp (56°C)	TGGTTGTCCAAGAAAAGAAA	CTATTCCCTTCTTACGTCCA	AJ002211
CCL2/ MCP-1	177 bp (65°C)	AGGAAGATCTCAGTGCAGAGG	AGTCTTCGGAGTTTGGGTTTG	X14768
CCL3/ MIP-1α	257 bp (65°C)	GCTGACTACTTTGAGACGAGC	CCAGTCCATAGAAGAGGTAGC	M23452
CCL5/ RANTES	583 bp (60°C)	GCTGTCCGTTTGATTTTTGTC	TGCTCATTCATCTTCTTTCTA	M21121

Table 3. RT-PCR Primers (cont.)

Probe	**Product length (bp) and Annealing temperature (°C)**	**Sense primer sequence**	**Antisense primer sequence**	**Accession no.**
CCL7/ MCP-3	216 bp (56°C)	ACTGAACTGAAAACAAGCCA	TCCAAGGCTTTATGTTCAAA	X72308
CCL17/ TARC	198 bp (56°C)	GGACCTGCACACACAGAGACT	GTCTGGTACCACGTCTTCAG	D43767
CCL20/ LARC/ MIP-3α	210 bp (56°C)	GATGTCAGTGCTGCTACTCC	TGGGTTCAGACGGGACCTAA	D86955
β-actin	176 bp (56°C)	CTTTTCCAGCCTTCCTCC	GCAGTAATCTCCTTCTGCATC	

Fig. 7 A schematic diagram of the RT-PCR technique



followed by 30 seconds at 72°C. Once cycling was completed, an extension phase was performed for 7 minutes at 72°C and the product was held at 4°C.

3.4.7 Detection of PCR products

A 2% agarose gel was made using 0.5xTBE (10mM Tris base, 10mM boric acid, 2 mM EDTA, pH 8.0). The agarose was dissolved by heating in a microwave, when cooled 1mg/ml of ethidium bromide was added. The gel was poured into a Bio-Rad gel tray and allowed to set. Five µl of sample loading buffer was added to each DNA sample. The samples and a lane of 100 base pair molecular weight markers were loaded onto the agarose-ethidium bromide gel and run by gel electrophoresis with 100 V/0.1 mA current (Bio-Rad instruments). The resulting bands were observed on an ultraviolet transilluminator and photographed using a polaroid camera and film.

3.5 IMMUNOHISTOCHEMISTRY STAINING OF GASTRIC MUCOSA

3.5.1 The patients

Patient consent was obtained and permission from the local Research Ethics Committee was granted before this study was carried out.

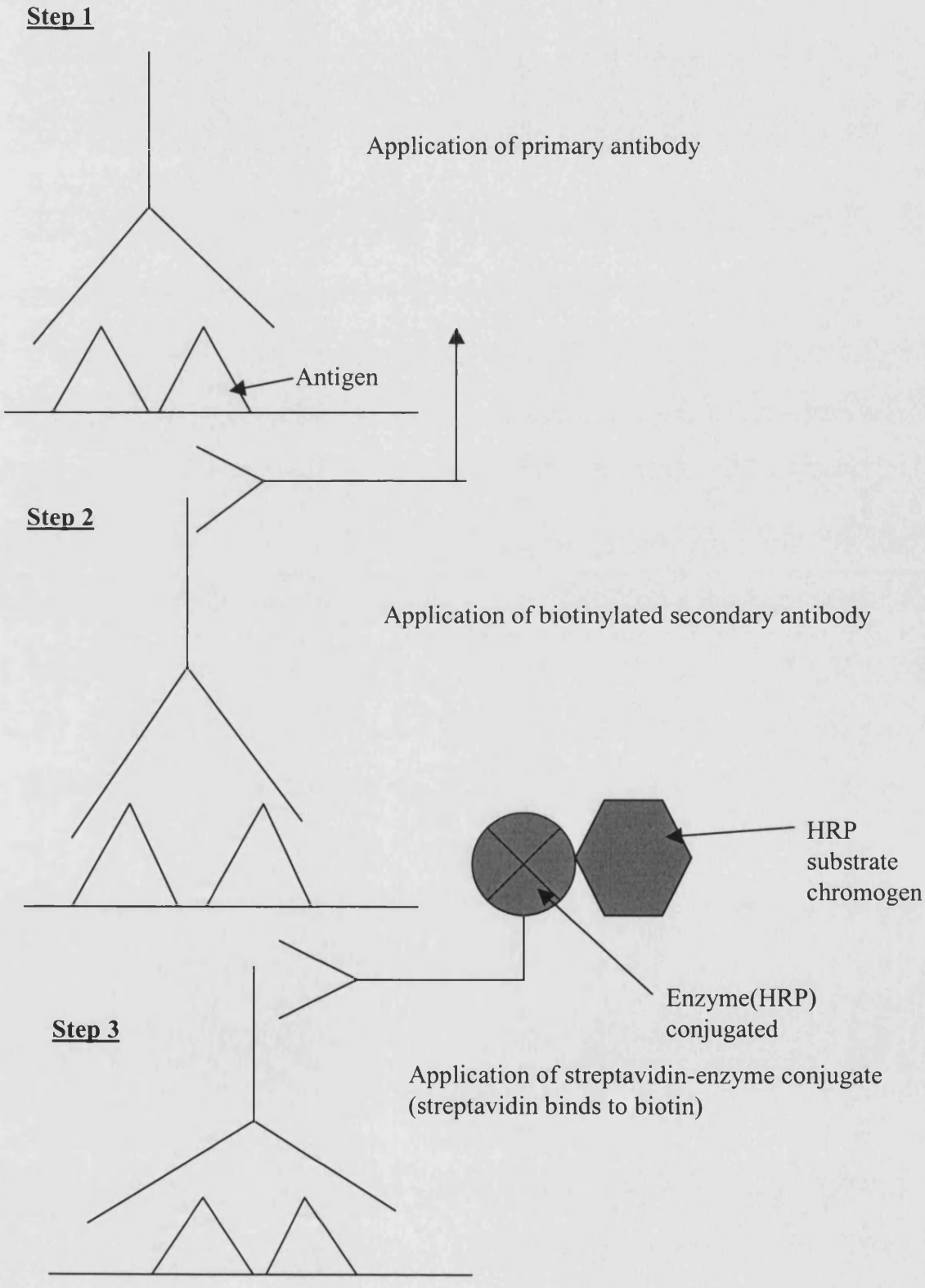
Samples of gastric mucosa were obtained from patients who had been examined via upper gastrointestinal endoscopy at the Royal National Hospital for Rheumatic Diseases (RNHRD), Bath. These patients had been referred by their general practitioners for a variety of gastrointestinal complaints. Some patients were diagnosed as normal when no visual pathological conditions were detected during the endoscopy and also if the 'CLO' test for the presence of *H. pylori* was negative. The other patient groups were patients who were diagnosed with gastritis and *H. pylori* infection and patients who had gastritis but no sign of *H. pylori* infection. Gastritis was diagnosed during the endoscopy when areas of inflammation in the stomach were detected.

Gastric biopsies were taken each from the antrum and body of the stomach. Generally, biopsies were collected from 4 normal patients, 6 patients with *H. pylori*-associated gastritis and 4 patients with non-*H. pylori*-associated gastritis. The numbers of biopsies collected from each patient depended on their pathological presentation. For the purpose of this study, 3-4 small pieces of gastric biopsies from the antrum and the body were collected and quickly collected in HBSS medium (pH 7.3) on ice for later RNA extraction and PCR analysis. The HBSS medium contained 100U/ml of penicillin, 100μg/ml of streptomycin, 50μg/ml of gentamycin, 2.5μg/ml of Amphotericin B/Fungizone®, Hepes buffer and sodium bicarbonate. For diagnostic purposes, 1 piece of gastric biopsy from the antrum was placed into the medium of the 'CLO' test to determine the presence of the bacteria *H. pylori*. The 'CLO' test works on the principle that the urease enzyme released by the bacteria converts the urea on the test medium into ammonia which results in a change in the colour of the phenol red present in the medium. The medium of the test would remain yellow if *H. pylori* was not present and become red in colour when there were *H. pylori* present in the specimen. When more detailed investigations were required for certain patients, extra biopsies were taken, usually 5-6 pieces which were put into a small plastic container containing formalin to be sent to the pathology laboratory at the Royal United Hospital(RUH) in Bath. Slides of gastric biopsies embedded in paraffin from the biopsy specimens were provided by the pathology laboratory for use in our laboratory. Some of these slides were later used in our laboratory for immunohistochemical analysis for the chemokines SDF-1α/CXCL12, BCA-1/CXCL13 and LARC/CCL20 and the chemokine receptor CXCR4.

3.5.2 Immunostaining for the chemokine receptor, CXCR4 and chemokines LARC/CCL20, SDF-1α/CXCL12 and BCA-1/CXCL13

Immunohistochemistry was performed on histological slides with paraffin-embedded gastric biopsies using the DAKO LSAB®2 System, based on a modified labelled avidin-biotin (LAB) technique in which a biotinylated secondary antibody forms a complex with peroxidase–conjugated streptavidin molecules (DAKO) (Fig.8). All recipes for buffers and solutions are found in section 3.8.5.

Fig.8 A schematic diagram of the avidin-biotin technique in immunohistochemistry



Mouse monoclonal antibody against the human CXC chemokine receptor, CXCR4 and chemokine LARC/CCL20, goat polyclonal antibody against the human chemokine BCA-1/CXCL13 and rabbit polyclonal antibody against the human chemokine SDF-1α/CXCL12 were used in an avidin-biotin method to demonstrate the presence of CXCR4/LARC(CCL20)/SDF-1α(CXCL12)/BCA-1(CXCL13) on the gastric biopsy histological slides.

Gastric biopsy sections which had been fixed in formalin and embedded in paraffin were mounted on APES coated slides which were supplied by the pathology laboratory in RUH. The mounted slides were deparaffinised twice with Citroclear for 5 minutes each time. Slides were washed twice in 99% industrial methylated spirit for one minute each time. Endogenous peroxidases within the embedded tissues were blocked with 2.5% hydrogen peroxide in methanol for 10 minutes and rehydrated in distilled water for 5 minutes. The slides were then washed with TBS and then heated in a pressure cooker for 2 minutes and soaked immediately in tap water to prevent the slide from drying. The primary antibody (mouse monoclonal antibody against human CXCR4) was diluted 1:75 in TBS and applied to the slide and left to incubate at room temperature for 45 minutes. The mouse monoclonal antibody against human LARC/CCL20 was diluted 1:10 in TBS and added to the slides which was incubated at room temperature for 45 minutes.

When the anti-human BCA-1/CXCL13 antibody was used, an extra step was introduced to improve the quality of the staining and to decrease non-specific staining in the background. After the slides were pressure-cooked for 2 minutes, the slides were rehydrated in water and then put into a humidified box. The DAKO-protein block serum free solution was applied neat to the slides for 20 minutes at room temperature. Subsequently, the goat polyclonal antibody against human BCA-1/CXCL13 was diluted 1:6 in 10ml TBS containing 1 drop of DAKO protein-block serum free liquid in it, to block non-specific binding. The antibody against human BCA-1/CXCL13 was applied to slides in a humidified box and left in the refrigerator for 24 hours to incubate (Mazzuchelli et al., 1999). An alternative method of antigen retrieval was used on slides stained for LARC/CCL20. Instead of heating the slides for 2 minutes in a pressure cooker, the slides were subjected to enzyme digestion with 0.01% Pronase in TBS for 40 minutes in a humidified box which was put into

an oven heated to 40°C. Both methods were run concurrently and comparisons were made. On each occasion, a negative control slide was included containing a similar biopsy section to which primary antibodies were not added, and the slide was incubated in TBS alone for the same duration in time as the slides with the primary antibodies.

Colour was developed on all slides using the protocol provided by DAKO. Slides were rinsed gently with TBS and stained with the yellow solution (biotinylated link goat antibody containing anti-rabbit and anti-mouse immunoglobulins in PBS) from the LSAB kit and incubated for 10 minutes at room temperature. The slides were rinsed again with TBS and treated with the red solution (streptavidin-linked to horseradish peroxidase) also from the LSAB kit for 10 minutes before they were rinsed again with TBS. The slides were then stained with a mixture of 1 ml DAB buffer (imidazole-HCl buffer, pH 7.5 containing hydrogen peroxide and an anti-microbial agent) and 1 drop of chromogen (3,3'-diaminobenzidine in chromogen solution) and this mixture was left on the slide at room temperature for 7 minutes to allow colour development. The slide was then washed with tap water and immersed in haematoxylin for 30 seconds to 1 minute. After rinsing the slide with water to remove the excess haematoxylin, the slide was immersed twice for 1 minute each time in 99% industrial methylated spirit to dehydrate the sample. Finally, the slide was immersed twice in Citroclear for 3 minutes each time to remove the alcohol. Some mounting media was applied onto a coverslip and the sample on the slide was covered taking care not to trap any air bubbles against the sample. The slide was then left to dry for 30 minutes to 1 hour before it was examined under a light microscope.

3.5.3 Isolation of gastric epithelial cells from gastric biopsies

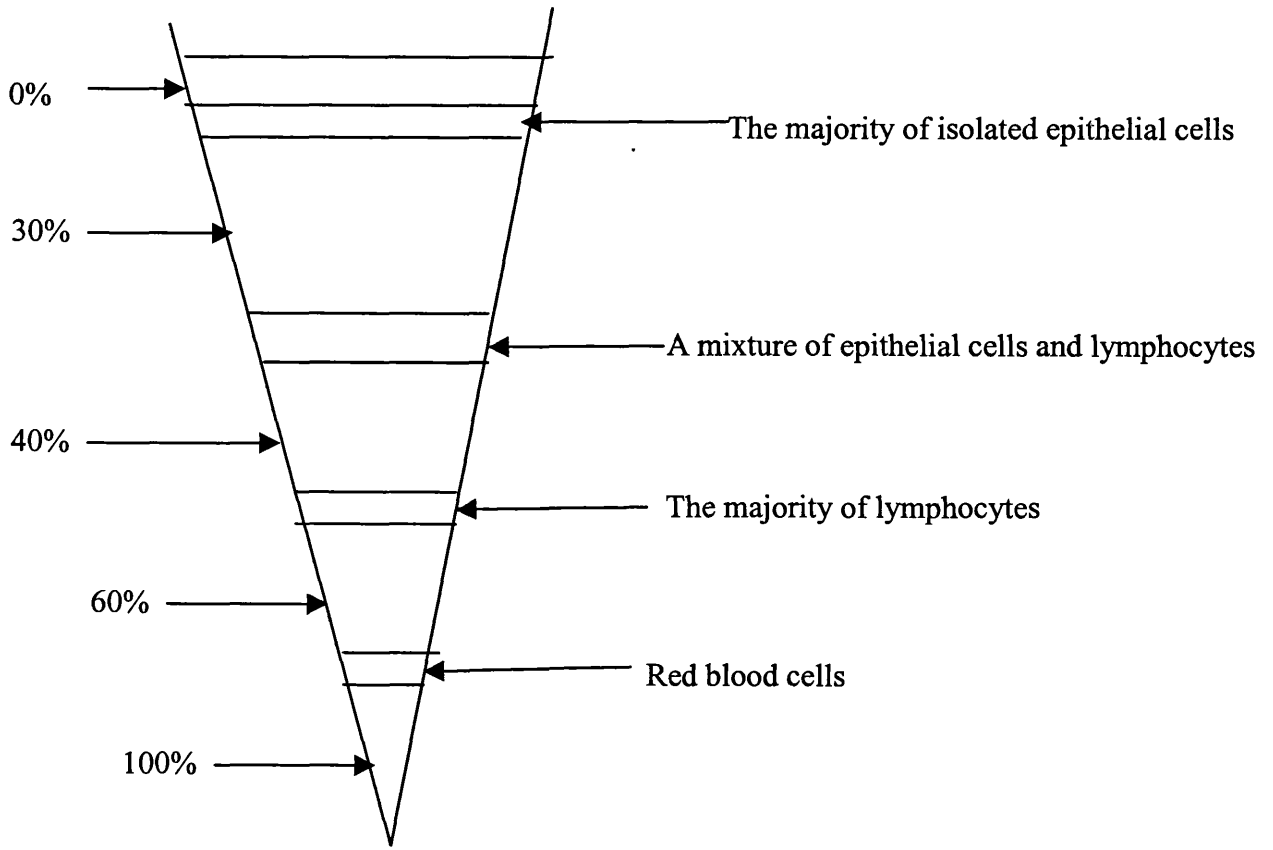
This method was obtained by direct communication from Dr. G. Kolios.

Small numbers of gastric epithelial cells were isolated under sterile conditions from gastric biopsies obtained during upper gastrointestinal endoscopies for possible analysis by RT-PCR. Three or four small pieces of gastric biopsies were collected in cold HBSS medium (pH 7.3) with added antibiotics and stored in ice. Within 1-2

hours after collection, the tissues were washed 3 times for 3-5 minutes each time using the cold HBSS medium. Following this, the tissues were minced carefully using sterile blades and washed in HBSS medium (pH 7.3) which were free from any Ca^{2+} and Mg^{2+} ions and contained antibiotics. The tissues were then transferred into a mixture of 15 ml of fresh HBSS medium free from any Ca^{2+} and Mg^{2+} ions and containing 1mM dithiothreitol (DTT) and were incubated at room temperature for 10 minutes. The tissues were then rinsed three times and were digested with 1mg/ml of Dispase which were added to the Ca^{2+} and Mg^{2+} free HBSS. This enzyme digestion step was carried out at 37°C for 30 minutes and the tube containing the tissues were vortexed every 10 minutes. At the end of this process, the tissues were dispersed using a 1ml Gilson pippette. The supernatants which contained the epithelial cells were then collected. The tissues were then subjected to the Dispase digestion once more. Following this, the supernatants from both Dispase treatments were added together and centrifuged at 20° C for 10 minutes at 200g. The pelleted cells were resuspended in complete RPMI medium and counted. The cell mixture was then centrifuged again.

The pelleted cells were then resuspended in a conical tube containing 3ml layers of Percoll (pH 7.4) with increasing density gradients from 0% at the top, followed by 30%, 40%, 60% and 100% at the bottom (Fig.9). The Percoll density gradient was prepared by diluting the stock Percoll with RPMI complete medium. The Percoll density gradient was used to separate the epithelial cells from the other cells which were of different densities. Epithelial cells which are the least dense should be present at the 0%-30% interphase, a mixture of small epithelial cells and big lymphocytes collect at the 30%-40% interphase, the majority of lymphocytes collect at the 40%-60% interphase and red blood cells(erythrocytes) which are the most dense are found at the 60%-100% interphase. Cells layered on the Percoll density gradient in the conical tube was centrifuged at 500g for 30 minutes at 4°C. A small number of epithelial cells which were at the 0%-30% interphase of the Percoll gradient were collected and centrifuged for 10 minutes at 4°C and 200g. The pelleted epithelial cells were then resuspended in complete RPMI medium and counted before they were seeded into a small, sterile petri dish and incubated at 37°C. Isolating enough cells from the small pieces and small numbers of biopsies for

Fig. 9 A schematic diagram of the Percoll density gradient used to isolate gastric epithelial cells



further experimentation was difficult. The epithelial cell yield should have been greater had it been possible to use gastric surgical specimens.

3.6 Statistical Analysis

Each experiment was carried out 2-3 times. In the ELISA experiments, the samples were run in duplicate on each ELISA plate. The samples were subjected to a one-way ANOVA (analysis of variance) followed by a Dunnett's test which compared the results per group to the controls. The data were shown as the mean ± the standard error of the mean (SEM). Results which had a probability value of less than 0.05 ($p<0.05$) were taken to be statistically significant. It should be noted that the ELISA data is unlikely to come from a normal population and hence the validity of the statistical analysis is low.

3.7 MATERIALS

Table 4. A list of materials used and their sources

MATERIAL	SOURCE
Absolute ethanol	Hayman Ltd.(Witham ,UK)
Acetic acid	Aldrich (Gillingham, UK)
Agarose	Gibco BRL (Paisley, UK)
Amphotericin B (Fungizone)®	Gibco BRL (Paisley, UK)
BCA-1/CXCL13: Goat anti-human polyclonal antibody (diluted in TBS to make 0.5mg/ml stock)	R & D Systems (Abingdon,UK)
Blocking reagent (for Northern analysis)	Roche (Lewes, UK)
Boric acid	Sigma (Poole, UK)
Bovine serum albumin (BSA)	Sigma (Poole, UK)
Bromophenol blue	BDH (Poole, UK)
Cell culture plastics	Nunc/Life Technologies (Paisley, UK)
Chloroform	Fisons (Loughborough, UK)
Citric acid monohydrate	Sigma (Poole, UK)
Citroclear	HD Supplies (Poole, UK)
Cold trypsin	BioGenex (Berkshire, UK)
CSPD	Roche (Lewes, UK)
CXCR4 (Fusin): Purified mouse anti-human monoclonal antibody (diluted in TBS to make 1mg/ml stock)	Pharmingen (Oxford, UK)
DAB substrate chromogen solution (3,3-diaminobenzidine in chromogen solution)	DAKO (Ely, UK)
Diethyl pyrocarbonate (DEPC)	Sigma (Poole, UK)
5' Digoxigenin labelled probes (cocktails containing 3 and 4 antisense 30-mer oligonucleotides for IL-8, RANTES and β-actin)	R & D Systems (Abingdon, UK)

MATERIAL	SOURCE
Digoxigenin chemiluminescent detection kit for Northern blotting	Roche (Lewes, UK)
Dimethyl sulphoxide (DMSO)	Sigma (Poole, UK)
Dispase	Gibco BRL (Paisley, UK)
Dithiotreitol (DTT)	Sigma (Poole, UK)
DNA (100 bp) ladder	Gibco BRL (Paisley, UK)
DNAse I	Gibco BRL (Paisley, UK)
DNAse reaction buffer	Gibco BRL (Paisley, UK)
dNTP	Roche (Lewes, UK)
Ethanol	Fisher Scientific (Loughborough, UK)
Ethidium bromide	Sigma (Poole, UK)
Ethylenediaminetetraacetic acid (EDTA)	Sigma (Poole, UK)
Expand polymerase	Roche (Lewes, UK)
Ficoll	Sigma (Poole, UK)
Filter paper (Whatman)	Whatman (Cambridge, UK)
Filter tips (10,20,200 µl)	Greiner (Gloucestershire, UK)
Foetal bovine serum (FBS)(heat-inactivated)	Gibco BRL (Paisley, UK)
Formaldehyde	BDH (Poole, UK)
Formamide	BDH (Poole, UK)
Glacial acetic acid	Fisons (Loughborough, UK)
Glycerol	Fisher Scientific (Loughborough, UK)
Goat anti-mouse peroxidase conjugate	DAKO (Ely, UK)
Goat anti-rabbit peroxidase conjugate	DAKO (Ely, UK)
Haematoxylin/eosin stains	SurgiPath (Bretton, UK)

MATERIAL	SOURCE
Ham's F12 Nutrient mixture medium	Gibco BRL (Paisley, UK)
Hank's balanced salt solution (HBSS)(pH 7.3)	GibcoBRL (Paisley, UK)
Hepes buffer	Gibco BRL (Paisley, UK)
Hydrochloric acid	Fisher Scientific (Loughborough, UK)
Hydrogen peroxide (30% v/v solution)	Sigma (Poole, UK)
H_2SO_4	Fisher Scientific (Loughborough, UK)
IFN-γ: human recombinant protein; specific activity > 2 X 10^7 U/mg (diluted in sterile PBS + 0.1% (w/v) BSA and stored in 10µl aliquots (10^6U/ml) at –70° C)	Roche (Lewes, UK)
IL-1α: human recombinant protein; specific activity > 1 X 10^9 U/mg (diluted in sterile PBS + 0.1% (w/v) BSA and stored in 20µl aliquots (10µg/ml) at –20° C)	PeproTech (London, UK)
IL-4: human recombinant protein; specific activity > or = 2 X 10^6 U/mg (diluted in sterile PBS + 0.1% (w/v) BSA and stored in 20µl aliquots (100µg/ml) at -20°C)	PeproTech (London, UK)
Anti-IL-8/CXCL8 antibody: mouse anti-human monoclonal antibody (ELISA coating antibody) (diluted in PBS and stored in 20µl (2µg/ml) aliquots at -20°C)	R & D Systems (Abingdon, UK)
Anti-IL-8/CXCL8 antibody: biotinylated mouse anti-human monoclonal antibody (ELISA detecting antibody) (diluted in PBS and stored in 4µl aliquots (20ng/ml) at -20°C	R & D Systems (Abingdon, UK)

MATERIAL	SOURCE
IL-13: purified protein from culture supernatants of stable transfected CHO cells (Minty et al., 1993) (diluted in sterile PBS + 0.1% (w/v) BSA and stored in 5µl aliquots (5µg/ml) at -70°C)	Gift from Dr. A. Minty (Sanofi Recherche, Labege, France)
Industrial methylated spirits (IMS) 99%	Fisher Scientific (Loughborough, UK)
LSAB kit for immunohistochemistry	DAKO (Ely, UK)
Maleic acid	Sigma (Poole, UK)
Methanol	Fisons, Loughborough (UK)
Anti-human MIP-3α/LARC/CCL20 antibody: Mouse anti-human antibody (reconstituted in PBS to make a stock solution of 1mg/ml and stored at -20°C)	R & D Systems (Abingdon, UK)
MOPS (3-(N-morpholino)-propane-sulfonic acid))	Sigma (Poole, UK)
Nuclease-free water	Promega (Southampton, UK)
Nylon membranes (positively charged)	Roche (Lewes, UK)
Oligo $(dT)_{12-18}$ primer	Roche (Lewes, UK)
OPD tablets (o-phenylene-diamine (dihydrochloride)	Sigma (Poole, UK)
Pastettes, 3 & 9 inches	Alpha lab (Hampshire, UK)
PCR buffer + Mg^{2+}	Roche (Lewes, UK)
PCR tubes	Anachem (Luton, UK)
PCR primers (synthesised according to sequences provided)	Gibco BRL (Paisley, UK)
Percoll	Amersham Pharmacia Biotech (Little Chalfont, UK)
PHA-L (leucoagglutinin)	Sigma (Poole, UK)
Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}	Gibco BRL (Paisley, UK)
Plastic 7ml pots with lids	BIBBY Sterilin (Staffordshire, UK)

MATERIAL	SOURCE
Plastic 60 & 100 ml pots with lids	BIBBY Sterilin (Staffordshire, UK)
PMA(phorbol 13-myristate 12-acetate)	Sigma (Poole, UK)
Polaroid film (type 55)	Sigma (Poole, UK)
Pronase	DAKO (Ely, UK)
Propan-2-ol	Fisons (Loughborough,UK)
Anti RANTES/CCL5 antibody: mouse anti-human monoclonal antibody (RANTES coating antibody) (diluted in PBS and stored in 20µl aliquots (500µg/ml) at -20°C)	R & D Systems (Abingdon, UK)
Anti RANTES/CCL5 antibody: biotinylated goat anti-human polyclonal antibody (RANTES detecting antibody) (diluted in PBS and stored in aliquots of 10µg/ml at -20°C)	R & D Systems (Abingdon, UK)
Protein block-serum free liquid	DAKO (Ely, UK)
RNAsin	Promega (Southampton, UK)
RNAzol B®	Tel Test (Texas, USA)
RPMI 1640 medium	Gibco BRL (Paisley, UK)
Sarcosyl	BDH (Poole, UK)
SDF-1α: purified rabbit anti-human polyclonal antibody (diluted in TBS to make 1mg/ml stock)	Peprotech (London, UK)
Sodium acetate	Sigma (Poole, UK)
Sodium azide	Sigma (Poole, UK)
Sodium bicarbonate	Gibco BRL (Paisley, UK)
Sodium chloride	BDH (Poole, UK)
Sodium dodecyl sulfate (SDS)	Sigma (Poole, UK)
Sodium hydroxide	Sigma (Poole, UK)
Sodium sulphite	Sigma (Poole, UK)

MATERIAL	SOURCE
Streptavidin peroxidase (diluted in PBS and stored in aliquots at -20°C)	Sigma (Poole, UK)
Sucrose	BDH (Poole, UK)
Superscript	Pharmacia (Little Chalfont, UK)
TNF-α: human recombinant protein; specific activity 6X 10^7 U/mg (diluted in sterile PBS + 0.1% (w/v) BSA and stored in 100µl aliquots (100µg/ml) at -20°C)	Gift from Bayer (Slough, UK)
Trisodium citrate dihydrate	Aldrich (Gillingham, UK)
Trizma base	Sigma (Poole, UK)
Trypsin/EDTA solution	Gibco BRL (Paisley, UK)
Tween 20 (polyoxyethylene 20 sorbitan monolaurate)	Sigma (Poole, UK)
X-Omat Film	Amersham International (Little Chalfont, UK)

3.8 BUFFERS AND SOLUTIONS

3.8.1 Solutions and reagents for cell culture

Tissue culture reagents were (from Gibco unless stated) were used for cell cultures and all solutions were made using sterile MilliQ water.

Phosphate Buffered Saline (PBS), pH 7.4

140 mM NaCl
2.7 mM KCl
1.5 mM KH_2HPO_4
8.1 mM $Na_2HPO_4.12H_20$

Foetal calf/bovine serum (FCS/FBS)

FCS/FBS (Gibco) was heat-inactivated at 56°C and aliquoted and stored at -20°C.

HBSS (pH 7.3)

10 ml of HBSS (10X) (with calcium and magnesium)
0.6 ml of 7.5% sodium bicarbonate
2 ml of 1M HEPES
100 U/ml of Penicillin
100 μg/ml of streptomycin
2.5 μg/ml of Amphotericin B/Fungizone®
50 μg/ml of gentamicin

3.8.2 Solutions and buffers for Northern Analysis

DEPC-treated water or buffer

MilliQ water or buffer were treated with 1 ml of diethyl pyrocarbonate (DEPC, Sigma) per litre of water and incubated overnight at 37°C and autoclaved the following day for 20 minutes at 121°C. The DEPC was handled in the fume cupboard.

20% (w/v) SDS solution

20g SDS diluted in 100 ml sterile MilliQ water.

0.1% (w/v) SDS solution

20% (w/v) SDS diluted 1:200 using sterile MilliQ water

0.5 M EDTA stock solution, pH 8.0 (when diluted, used to make 20X MOPS running buffer)

0.5 M EDTA in MilliQ water, pH adjusted to 8.0 and autoclaved.

3 M Sodium acetate, pH 5.2 (when diluted, used to make 20X MOPS running buffer)

3 M Na acetate dissolved in 250 ml MilliQ water. pH adjusted to 5.2 with 3 M acetic acid. DEPC treated and autoclaved.

75% (v/v) Ethanol Solution

75 ml absolute ethanol added to 25ml of MilliQ water.

Ethidium bromide

10 mg ethidium bromide dissolved in 10 ml DEPC-treated water.
(stored in the dark)

20 X MOPS running buffer, pH 7.0

0.4 M MOPS
0.02 M EDTA 4 ml (from 0.5 M stock)
0.2 M Na acetate 6.64 ml (from 3 M stock)
Volume adjusted to 100 ml with MilliQ water and pH adjusted to 7.0 with solid NaOH.

1X MOPS running buffer

50 ml 20 X MOPS running buffer diluted with 950 ml DEPC-treated water.

Bromophenol blue solution

0.025 g bromophenol blue
3 ml glycerol
Volume adjusted to 10 ml with DEPC-treated water

20 X SSC, pH 7.0

3M NaCl
0.3M Na citrate or trisodium citrate dihydrate
Volume adjusted to 1 L with MilliQ water, pH adjusted to 7.0, DEPC-treated and autoclaved.

Buffer 1, pH 7.5

0.1 M maleic acid
0.15 M NaCl
pH adjusted to 7.5 with solid NaOH
Volume adjusted to 1 L with MilliQ water, DEPC-treated and autoclaved.

Blocking stock solution

10 g Boehringer blocking reagent in 100 ml buffer 1. Microwaved to dissolve (do not boil). Autoclaved.

Hybridisation solution

5 X SSC	25 ml (from 20 X SSC)
0.1% Sarcosyl	0.5 ml (from 20% stock)
0.02% SDS	0.1 ml (from 20% stock)
1% blocking buffer	10 ml (from 10% stock)

Volume adjusted to 100 ml with DEPC-treated water

2 X SSC , 0.1% SDS

50 ml of 20 X SSC
2.5 ml of SDS (from 20% stock)
Volume adjusted to 500 ml with DEPC-treated water.

0.1 X SSC, 0.1% SDS

2.5 ml of 20 X SSC
2.5 ml of SDS (from 20% stock)
Volume adjusted to 500 ml with DEPC-treated water.

Washing buffer

0.3% (v/v) in buffer 1.

Buffer 2

10% (v/v) blocking stock solution in buffer 1.

Buffer 3, pH 9.5

0.1 M TRIS
0.1 M NaCl
Volume adjusted to 500 ml with MilliQ water and pH adjusted to 9.5

3.8.3 Solutions and buffers for ELISA

Bovine serum albumin (BSA)

10% (w/v) in MilliQ
Stored in 1 ml aliquots at -20°C

Phosphate buffered saline (PBS), pH 7.4

Same as for section 2.9.1

Wash buffer

1 L of PBS, pH 7.4
0.5 ml of Tween 20
(stored at 4-8°C)

Blocking buffer

1% BSA
5% sucrose
0.05% NaN_3
Filter sterilised and frozen in aliquots of 9.5 ml

Tris buffered saline (TBS), pH7.3

20mM of TRIZMA base
150mM of NaCl
MilliQ added to the specified volume, pH adjusted to 7.3

Dilution buffer

0.5 ml of 10% BSA stock
25 µl of Tween 20
TBS added till the volume 50 ml

Streptavidin peroxidase

1:2000 dilution of 1 mg/ml stock in dilution buffer

Substrate buffer, pH 5.0

7.3 g of citric acid monohydrate
9.46 g of Na_2HPO_4
Volume adjusted with MilliQ water to 1 L, pH adjusted to pH 5.0

Substrate solution

50 ml of substrate buffer (37°C)
1 OPD tablet
20 µl hydrogen peroxide

Stopping solution

27 ml of concentrated H_2SO_4 made up to 500 ml with MilliQ water

3.8.4 Solutions and buffers for RT-PCR

5 X TBE stock

54 g of Trizma base
27.5 g of boric acid
20 ml of EDTA (pH 8.0)
Volume adjusted to 1 L

Sample loading buffer

0.25% bromophenol blue
15% Ficoll (type 400) in water

3.8.5 Solutions and buffers for immunohistochemistry

Blocking reagent

5 ml of hydrogen peroxide
200 ml of methanol

Tris buffered saline (TBS), pH 7.6

50 mM Tris
150 mM NaCl
pH adjusted to 7.6 with HCl

Citrate buffer

29.4 g sodium citrate

27 ml 2M HCl

Volume made up to 10 L and pH adjusted to pH 6.0

4.0 RESULTS

4.1 CHEMOKINE mRNA EXPRESSION BY AGS AND MKN45 GASTRIC EPITHELIAL CELL LINES IN RESPONSE TO CYTOKINE STIMULATION

In the following results section, the expression and regulation of chemokines have been investigated by Northern analysis in the gastric epithelial cell lines AGS and MKN45, which are models of 'real' gastric epithelial cell lines. These cell lines have been used to investigate chemokine expression in place of real isolated gastric epithelial cells which are difficult to isolate in large numbers and when they are isolated, they are difficult to maintain in culture as they rapidly undergo apoptosis (programmed cell death). Northern analysis of chemokine expression was attempted on freshly isolated human gastric epithelial cells, but due to low cell numbers and rapid cell death, it was not possible to obtain sufficient RNA for chemokine analysis.

IL-8/CXCL8 mRNA has been investigated as an example of the neutrophil chemoattractant from the 'CXC' chemokine family and RANTES/CCL5 was studied as a T cell chemoattractant from the 'CC' chemokine family. Pro-inflammatory cytokines, IL-1α, TNF-α and IFN-γ were chosen as stimulants used to activate the cells as they are produced by immune cells infiltrating the gastric epithelium during infections with *H. pylori* (Wilson et al., 1998) or during other inflammatory conditions. These cytokines have previously been shown in this laboratory to induce chemokines in a number of non-immune cell types including fibroblasts, smooth muscle cells (Jordan et al., 1997), mesangial cells (Brown et al., 1994; Robson et al., 1995 (a); Robson et al., 1995(b)) and colonic epithelial cells (Kolios et al., 1999).

4.1.1 IL-8/CXCL8 mRNA expression

All experiments were performed in growth arrested cells which had been cultured in Ham's F12 nutrient medium for the AGS cells or RPMI medium for the MKN45 cells in the absence of foetal bovine serum overnight prior to the experiment and throughout the course of the experiment.

Confluent monolayers of AGS cells were stimulated with IL-1α (3ng/ml), TNF-α (30ng/ml) and IFN-γ (100U/ml) alone or in combination. Experimental controls were cells unstimulated and maintained in serum-free medium throughout the course of the experiment. When the three cytokines were added together, this combination was called 'cytomix'. The concentrations used in the present study have previously been shown in this laboratory to cause maximum stimulation of chemokine expression in a number of different 'non-immune' cell types (Brown et al., 1994; Robson et al., 1995; Jordan et al., 1997; Kolios et al., 1999). The experiments were carried out at these time intervals 1, 2, 4, 6, 12, 24 and 48 hours to enable a pattern of chemokine expression to be determined.

AGS

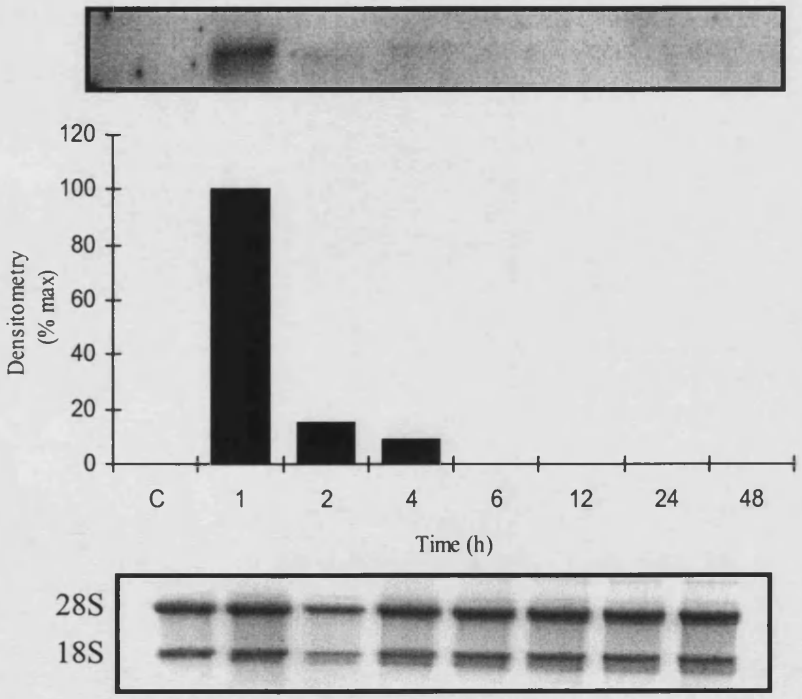
Detectable IL-8/CXCL8 mRNA was not expressed constitutively by the AGS cell line incubated with medium alone. In the AGS cell line, stimulation with IL-1α (3ng/ml) alone resulted in a time-dependent IL-8/CXCL8 mRNA expression with maximum detection 1 hour post-stimulation, which then decreased rapidly. Expression of IL-8/CXCL8 mRNA was short-lived in response to IL-1α (Fig.10). As shown in Fig.11, TNF-α(30ng/ml) induced a more prolonged expression of IL-8/CXCL8 mRNA in AGS cells, which was still detectable up to 24 hours post-stimulation, but levels had decreased. IL-1α(3ng/ml) and TNF-α(30ng/ml) added in combination, induced an early maximum expression at 2 hours post-stimulation and the expression was detectable at high levels until 6 hours following which the expression decreased (Fig.12).

IFN-γ(100U/ml) alone did not induce IL-8/CXCL8 mRNA expression (results not shown) and did not affect IL-1α(3ng/ml) or TNF-α(30ng/ml) response in combination (Figs. 13 & 14).

IFN-γ(100U/ml), TNF-α(30ng/ml) and IFN-γ(100U/ml) combination (Fig.15) induced IL-8/CXCL8 mRNA expression rapidly with a maximum expression at 1-2 hours followed by a decrease by 48 hours. The combination resulted in low levels but

Fig. 10

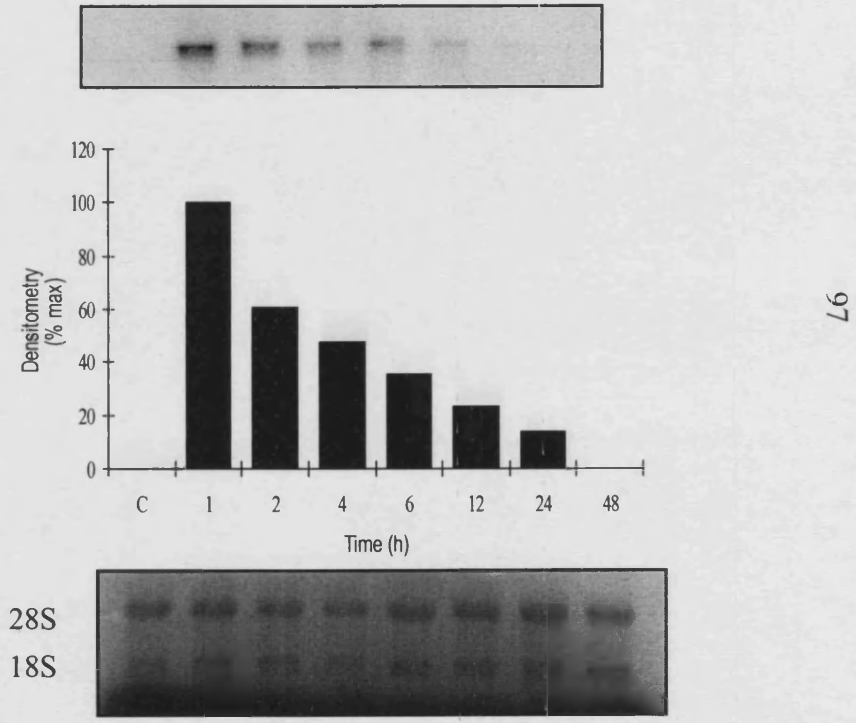
Time course of the expression of IL-8 /CXCL8 mRNA by AGS cells stimulated with IL-1α (3ng/ml)



AGS cells were stimulated with IL-1α (3ng/ml) from 1-48 hours. 'C' represents unstimulated control cells which were incubated in medium only. At the appropriate times the cells were lysed into 'RNAzol B'. RNA was extracted and run on 1% agarose/formaldehyde gels. IL-8 mRNA was detected by Northern analysis (top panel). Relative amounts of IL-8 mRNA were measured by scanning densitometry (middle panel). The bottom panel shows the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.11

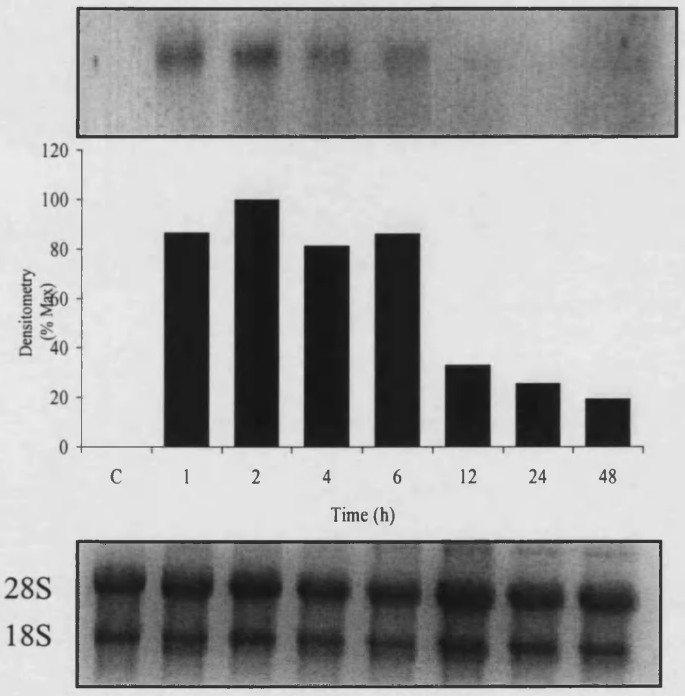
Time course of the expression of IL-8/CXCL8 mRNA by AGS cells stimulated with TNF-α (30ng/ml)



AGS cells were stimulated with TNF-α (30ng/ml) from 1-48 hours. 'C' represents unstimulated control cells incubated in medium only. At the appropriate times the cells were lysed into 'RNAzol B'. RNA was extracted and run onto a 1% agarose/formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). Relative amounts of IL-8 mRNA were measured by scanning densitometry (middle panel). The bottom panel represents the ethidium bromide- stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.12

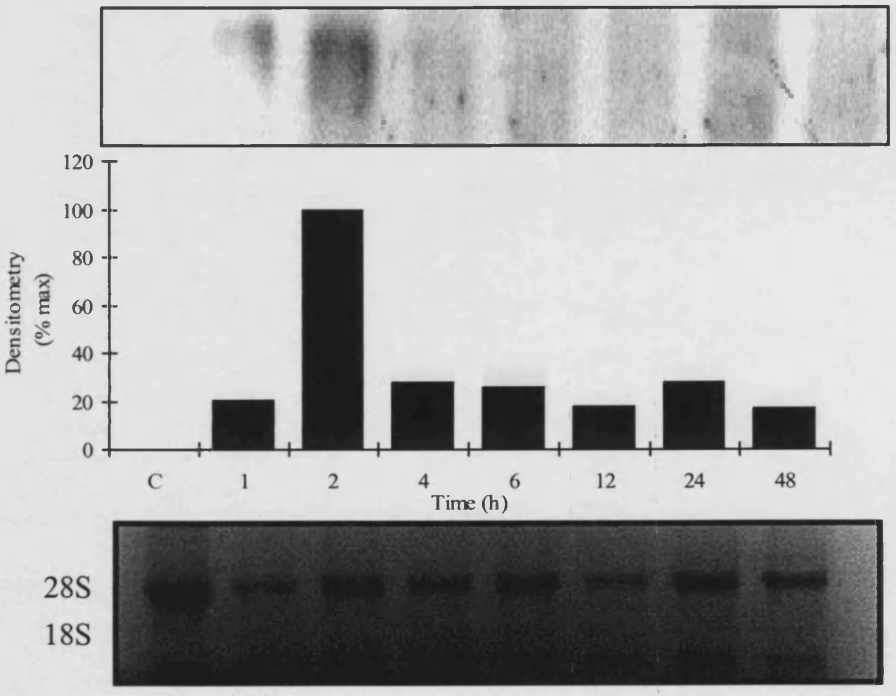
The time course of IL-8/CXCL8 mRNA expression by AGS cells stimulated with IL-1α (3ng/ml) and TNF-1α (30ng/ml)



AGS cells were stimulated with a combination of IL-1α and TNF-α from 1-48 hours. 'C' represents unstimulated control cells incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run onto a 1% agarose/formaldehyde gel. Relative concentrations of IL-8 mRNA was detected by scanning densitometry (middle panel). The bottom panel shows the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.13

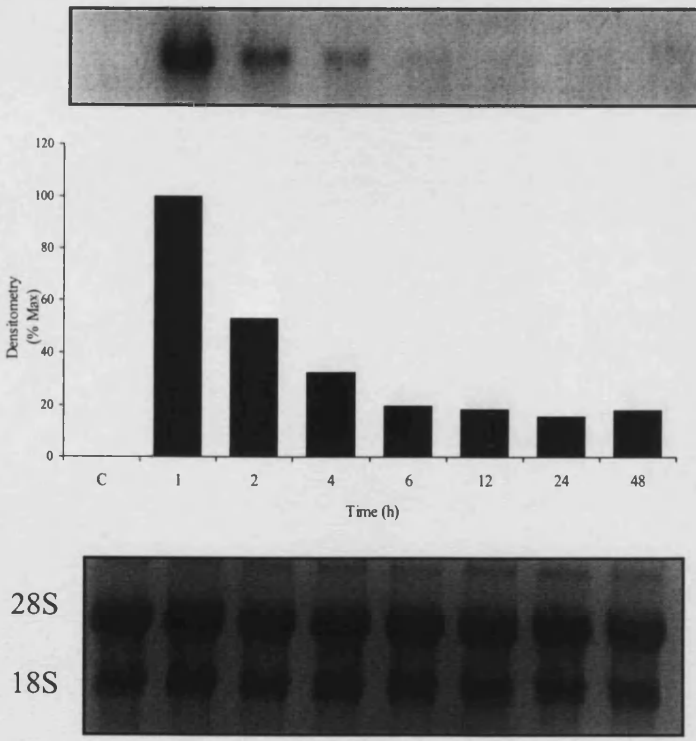
The time course of IL-8/CXCL8 mRNA expression by AGS cells stimulated with IL-1α (3ng/ml) and IFN-γ (100U/ml)



AGS cells were stimulated with IL-1α and IFN-γ from 1-48 hours. 'C' represents unstimulated control cells incubated in medium only. At the appropriate times the cells were lysed into 'RNAzol B'. RNA was extracted and run on 1% agarose/formaldehyde gels. IL-8 mRNA was detected by Northern analysis (top panel). Relative amounts of IL-8 mRNA were measured by scanning densitometry (middle panel). The bottom panel shows the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.14

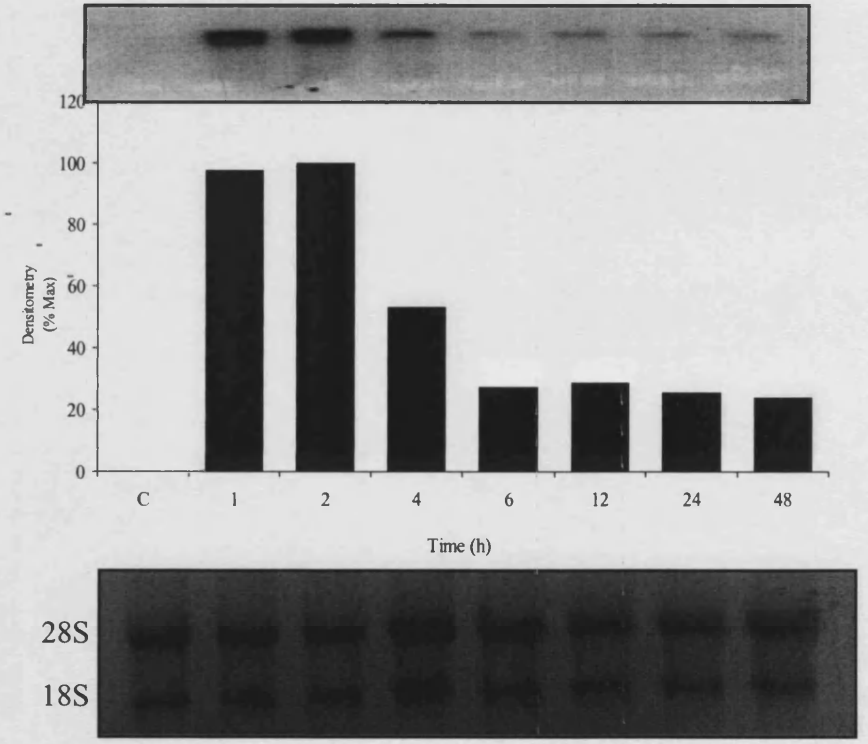
The time course of IL-8/CXCL8 mRNA expression by AGS cells stimulated with TNF-1α (30ng/ml) and IFN-γ (100U/ml)



AGS cells were stimulated with TNF-α and IFN-γ from 1-48 hours. 'C' represents unstimulated control cells which were incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run onto a 1% agarose/formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). IL-8 mRNA was detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.15

The time course of IL-8/CXCL8 mRNA expression by AGS cells stimulated with IL-1α (3ng/ml) , TNF-1α (30ng/ml) and IFN-γ (100U/ml)



AGS cells were stimulated with cytomix from 1-48 hours. 'C' represents unstimulated control cells which were incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run onto a 1% agarose/formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). IL-8 mRNA was detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

much more prolonged IL-8/CXCL8 mRNA expression than occurred with any of the stimulations individually.

MKN45 cells

MKN45 cells did not express IL-8/CXCL8 mRNA constitutively when the cells were incubated overnight with medium alone. This is indicated as "C" in all figures.

Stimulation with IL-1α(3ng/ml) resulted in rapid IL-8/CXCL8 mRNA expression which was very short-lived, with very low detectable levels between 2-24 hours (Fig.16). As was seen in the AGS cells, TNF-α(30ng/ml) induced a more sustained response in MKN45 cells lasting up to 6 hours post-stimulation. The maximum expression was still rapid occurring at 1 hour post-stimulation (Fig.17).

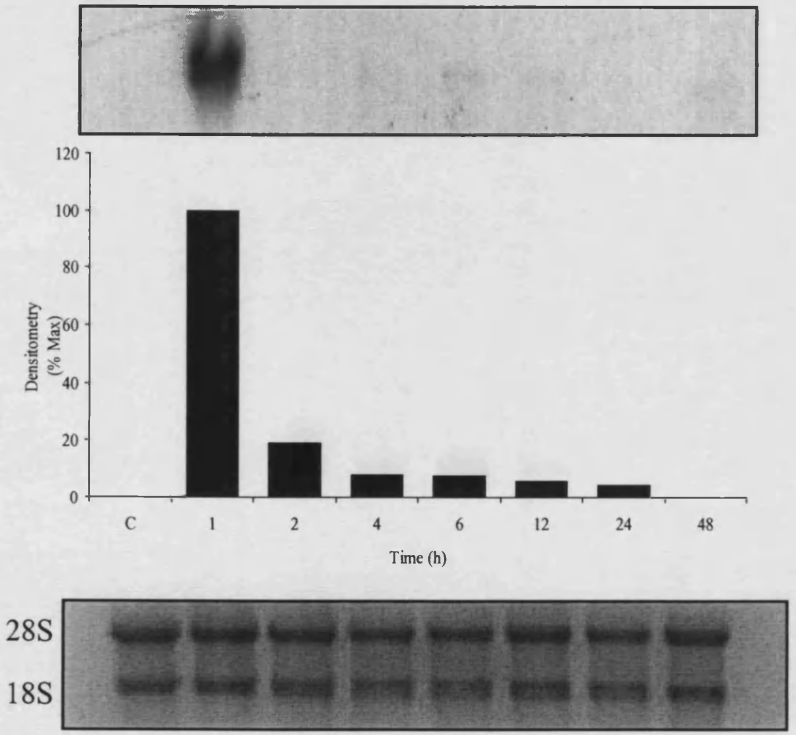
IL-1α(3ng/ml) and TNF-α(30ng/ml) added in combination induced high expression of IL-8/CXCL8 mRNA from 1-6 hours (Fig.18). Stimulation with IFN-γ alone did not induce IL-8/CXCL8 mRNA expression (results not shown). As shown in Fig.19, IFN-γ(100U/ml) added in combination with IL-1α(3ng/ml) induced a small prolonged expression of IL-8/CXCL8 mRNA than occured with IL-1α(3ng/ml) alone. Peak expression occured rapidly at 1 hour and response was not detected after 4 hours stimulation.

Combination TNF-α(30ng/ml) and IFN-γ(100U/ml) demonstrated a very similar result to TNF-α(30ng/ml) alone indicating that in this cell line, IFN-γ(100U/ml) did not significantly regulate IL-8/CXCL8 mRNA expression (Fig.20).

Similarly, 'cytomix' stimulation resulted in IL-8/CXCL8 mRNA expression as seen with IL-1α(3ng/ml) and TNF-α(30ng/ml) in combination, again suggesting that IFN-γ(100U/ml) does not have an effect (Fig.21).

Fig.16

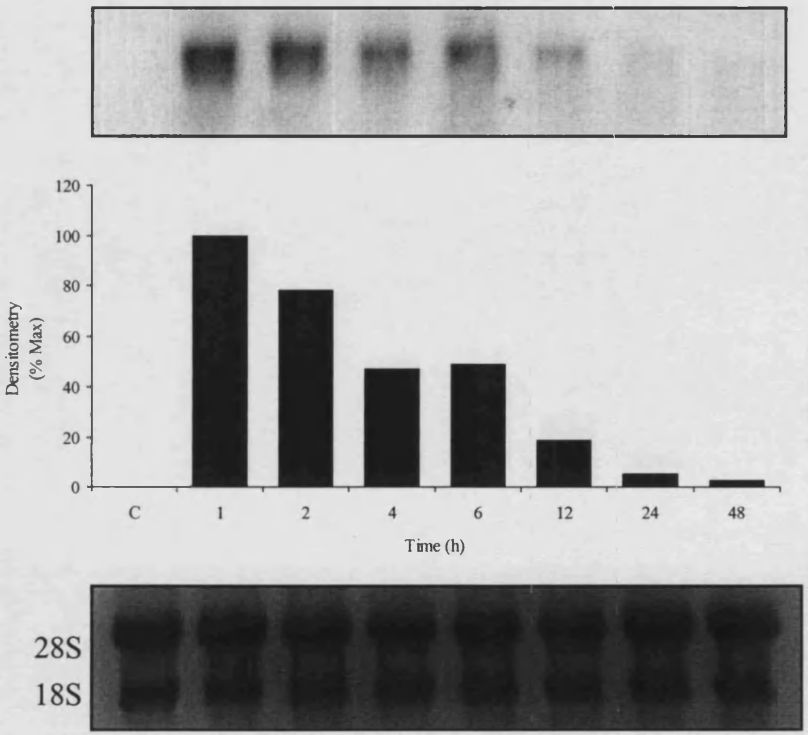
The time course of IL-8/CXCL8 mRNA expression by MKN45 cells stimulated with IL-1α (3ng/ml)



MKN45 cells were stimulated with IL-1α from 1-48 hours. 'C' represents unstimulated control cells incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run onto a 1% agarose/formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). Relative concentrations of IL-8 mRNA were detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.17

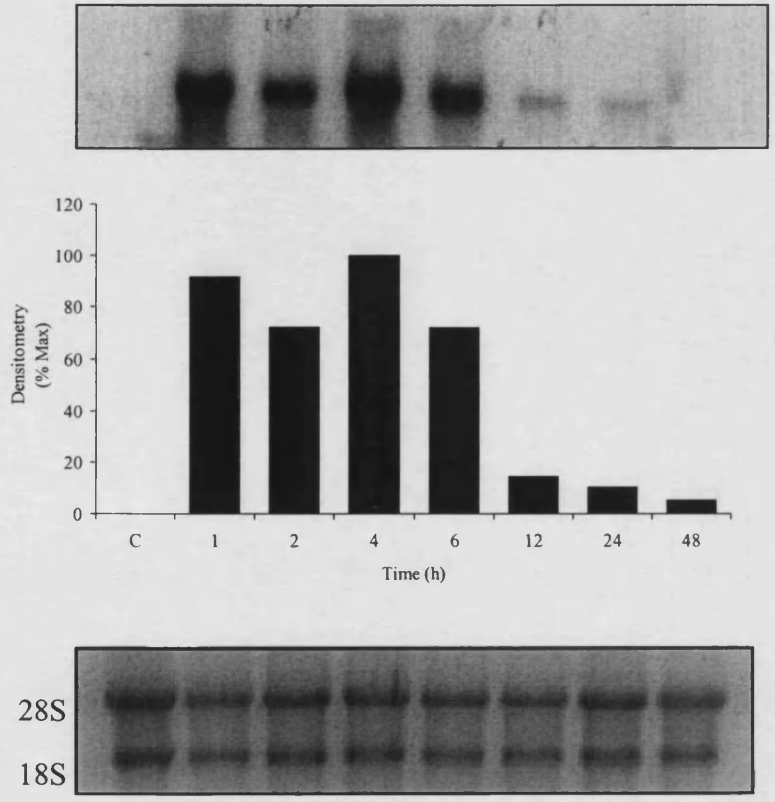
The time course of IL-8/CXCL8 mRNA expression by MKN45 cells stimulated with TNF-α (30ng/ml)



MKN45 cells were stimulated with TNF-α from 1-48 hours. 'C' represents unstimulated control cells incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run onto a 1% agarose/formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). Relative concentrations of IL-8 mRNA were detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.18

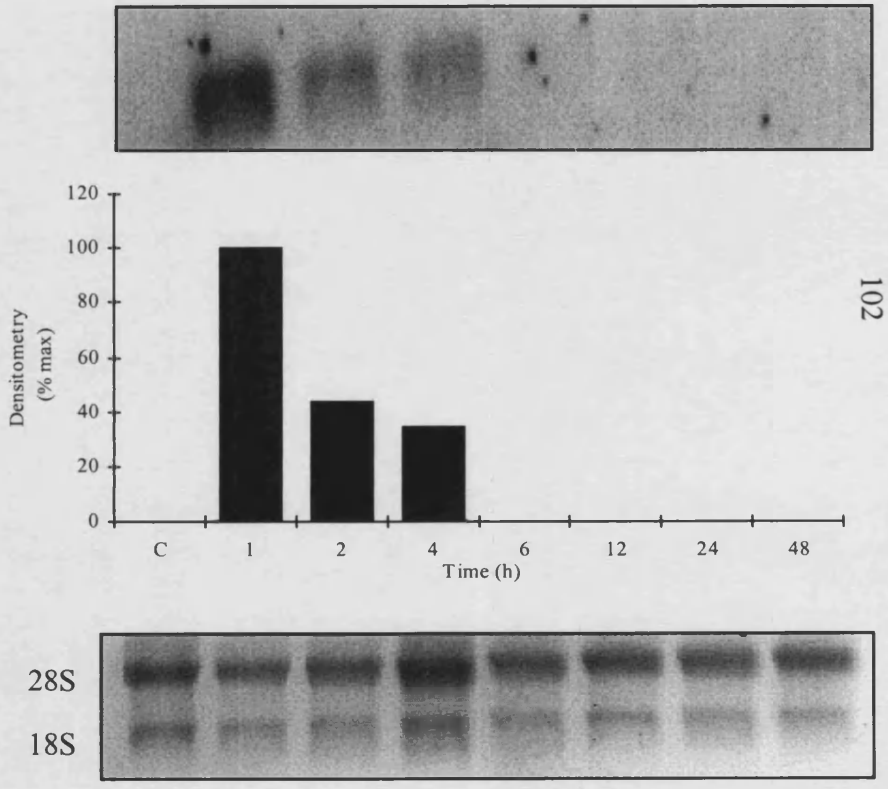
The time course for IL-8/CXCL8 mRNA expression by MKN45 cells stimulated with IL-1α (3ng/ml) and TNF-1α (30ng/ml)



MKN45 cells were stimulated with IL-1α and TNF-α from 1-48 hours. 'C' represents unstimulated control cell which were incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run on a 1% agarose/formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). Relative concentrations of IL-8 mRNA was detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading. Representative of three independent experiments.

Fig.19

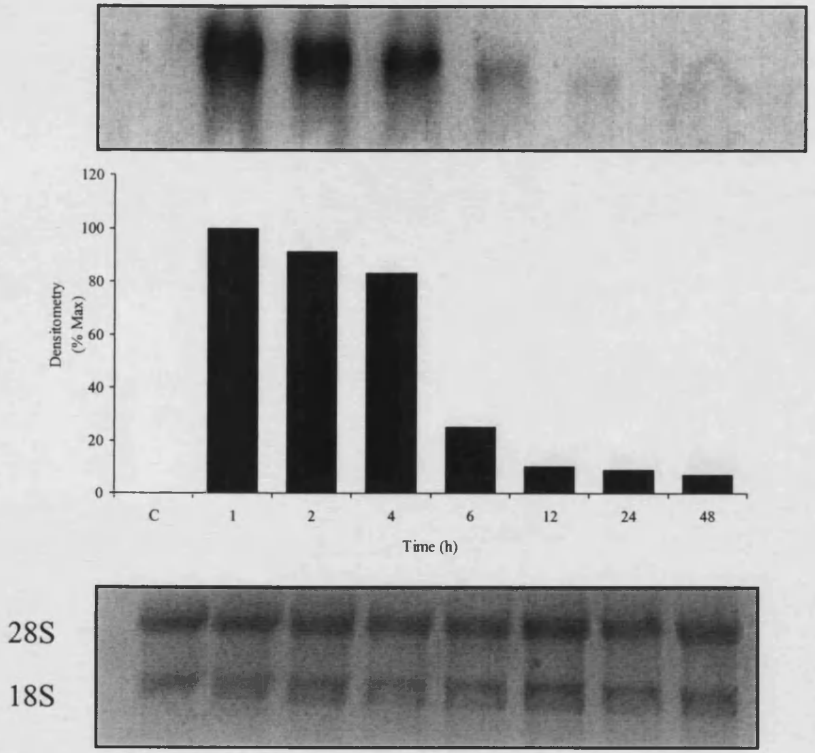
Time course of the expression of IL-8/CXCL8 mRNA expression by MKN45 cells stimulated with IL-1α (3ng/ml) and IFN-γ (100U/ml)



MKN45 cells were stimulated with IL-1α and IFN-γ from 1-48 hours. 'C' represents unstimulated control cells which were incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run on a 1% agarose /formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). Relative concentrations of IL-8 mRNA were detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading. Representative of three independent experiments.

Fig.20

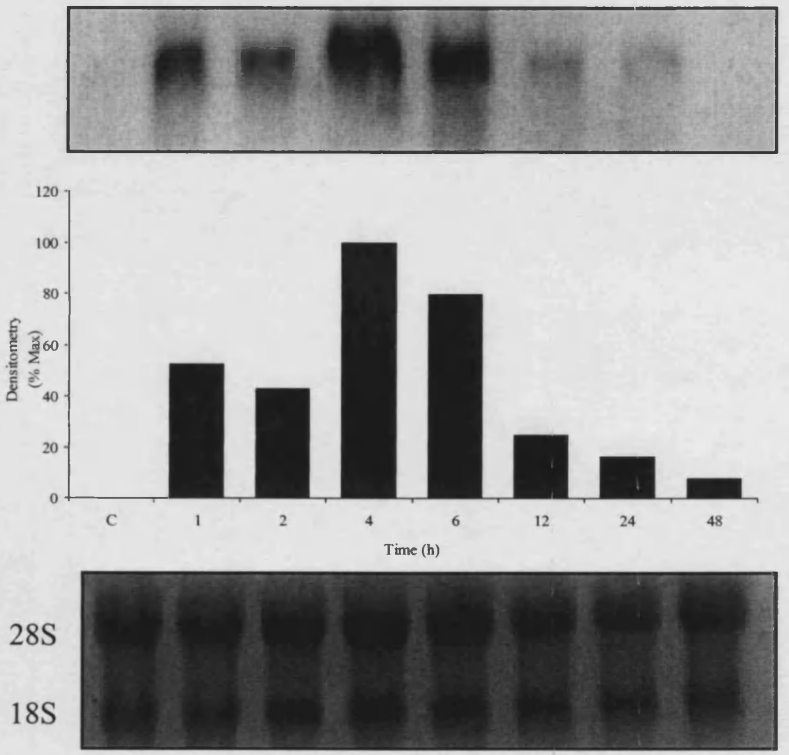
The time course for IL-8/CXCL8 mRNA expression by MKN45 cells stimulated with TNF-α (30ng/ml) and IFN-γ (100U/ml)



MKN45 were stimulated with TNF-α and IFN-γ from 1-48 hours. 'C' represents unstimulated control cells which were incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run on 1% agarose/ formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). Relative concentrations of IL-8 mRNA were detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.21

The time course for IL-8/CXCL8 mRNA expression by MKN45 cells stimulated with IL-1α (3ng/ml), TNF-α (30ng/ml) and IFN-γ (100U/ml)



MKN45 were stimulated with cytomix from 1-48 hours. 'C' represents unstimulated control cells which were incubated in medium only. At the appropriate times, the cells were lysed into 'RNAzol B'. RNA was extracted and run on 1% agarose/formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). Relative concentrations of IL-8 mRNA were detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

4.1.2 RANTES/CCL5 mRNA expression

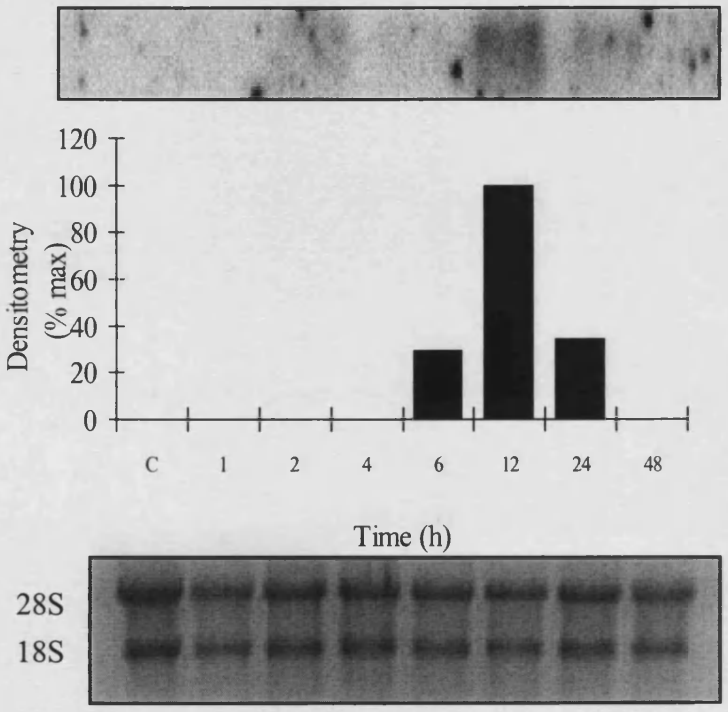
RANTES/CCL5 mRNA expression was investigated using Northern analysis on AGS cells stimulated in a number of experiments with IL-1α(3ng/ml), TNF-α (30ng/ml) and IFN-γ(100U/ml) alone and in all possible combinations from 1-48 hours. RANTES/CCL5 mRNA transcripts were not detected in the AGS cell line (results not shown).

MKN45 cells

In MKN45 cells, RANTES/CCL5 mRNA was detected. Requirements for the activation and time course of expression were very different from IL-8/CXCL8 mRNA expression. The minimum stimuli needed to induce expression of RANTES/CCL5 mRNA was TNF-α(30ng/ml) and IFN-γ(100U/ml) in combination (Fig.22). The only other stimuli which could induce RANTES/CCL5 mRNA expression was a combination of IL-1α(3ng/ml), TNF-α(30ng/ml) and IFN-γ(100U/ml) (Fig.23). In contrast to the expression of IL-8/CXCL8 mRNA expression, RANTES/CCL5 mRNA expression was also late in onset. RANTES/CCL5 mRNA was expressed from 6 hours post-stimulation with a peak at 12 hours. A combination of TNF-α(30ng/ml) and IFN-γ(100U/ml) with the addition of IL-1α(3ng/ml) did not induce a different RANTES/CCL5 mRNA expression indicating that IL-1α(3ng/ml) had no effect. Again, a late peak at 12 hours was detected.

Fig.22

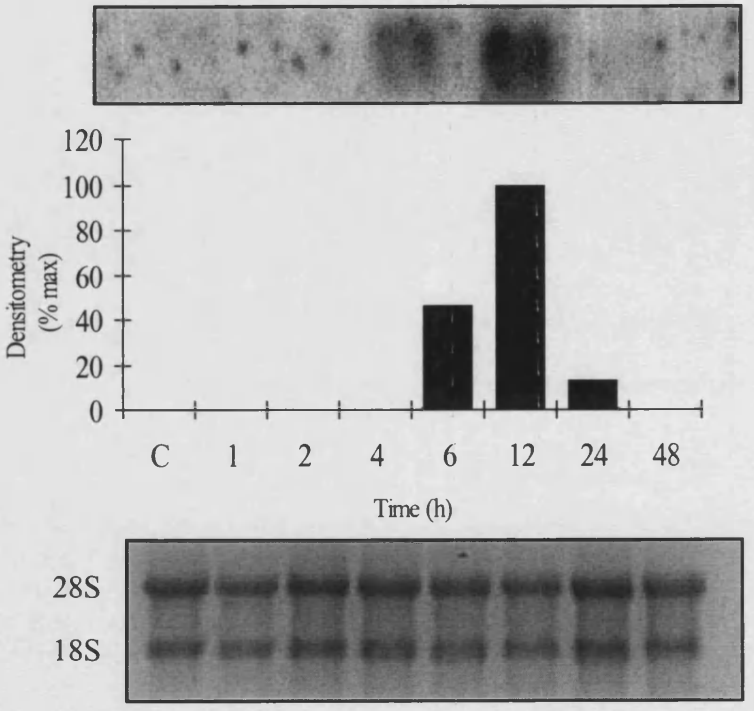
Time course of the expression of RANTES/CCL5 mRNA by MKN45 cells stimulated with TNF-α (30 ng/ml) and IFN-γ (100U/ml)



MKN45 cells were stimulated with TNF-α and IFN-γ from 1-48 hours. C represents unstimulated control cells which were incubated in medium only. At the appropriate times the cells were lysed into 'RNAzol B'. RNA was extracted and run on 1% agarose/formaldehyde gels. RANTES mRNA was detected by Northern analysis (top panel). Relative amounts of RANTES mRNA were measured by scanning densitometry (middle panel). The bottom panel shows the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.23

Time course of the expression of RANTES/CCL5 mRNA by MKN45 cells stimulated with IL-1α (3ng/ml), TNF-α (30ng/ml) and IFN-γ (100U/ml)



MKN45 cells were stimulated with IL-1α, TNF-α and IFN-γ for 1-48 hours. C represents unstimulated control cells which were incubated in medium only. At the appropriate times the cells were lysed into 'RNAzol B'. RNA was extracted and run on 1% agarose/formaldehyde gels. RANTES mRNA was detected by Northern analysis (top panel). Relative amounts of RANTES mRNA were measured by scanning densitometry (middle panel). The bottom panel shows the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

4.2 SECRETION OF THE IL-8/CXCL8 PROTEIN BY AGS AND MKN45 CELL LINES DETECTED BY ELISA

Enzyme-linked immunoassays (ELISA) were carried out to determine whether IL-8/CXCL8 and RANTES/CCL5 mRNA expressed by the AGS and MKN45 cells was translated into secreted protein. The chemokine protein was measured in supernatants collected from the cell cultures, after the cells had been stimulated with high concentrations of the cytokines. IL-8/CXCL8 secreted by the AGS and MKN45 cell lines which had been stimulated with IL-1α (10ng/ml) alone, TNF-α (100ng/ml) alone or 'cytomix' (300U/ml) were measured during a time course from 1-72 hours. Higher concentrations of the pro-inflammatory cytokines were used in the ELISA experiments compared to those used in the Northern analysis experiments. This was to ensure that maximum levels of IL-8/CXCL8 and RANTES/CCL5 protein were secreted by these cell lines in anticipation of possible down-regulation by anti-inflammatory cytokines (IL-4 or IL-13) which would be used together with the pro-inflammatory cytokines in the subsequent experiments. RANTES/CCL5 protein secretion expression by the MKN45 cells were also measured during a time course from 1-72 hours when the MKN45 cells were stimulated with TNF-α(100ng/ml) and IFN-γ(300U/ml) in combination or 'cytomix', which was made up of IL-1α(10ng/ml), TNF-α(100ng/ml) and IFN-γ(300U/ml).

4.2.1 Secretion of IL-8/CXCL8 protein by the AGS cells stimulated with IL-1α(10ng/ml) or TNF-α (100ng/ml) or 'cytomix' during a time course

IL-8/CXCL8 protein was not secreted constitutively by unstimulated AGS cells. However, stimulation with either one of the cytokines or 'cytomix' indicated that very low levels of secreted IL-8/CXCL8 could be detected as early as 1 hour post-stimulation (Fig.24). Very similar levels were secreted at 1 hour post-stimulation with IL-1α which was 0.61 ng/ml and 0.34 ng/ml when the AGS cells were stimulated with TNF-α. The expression in IL-8/CXCL8 increased up to 24 and 48 hours, by a similar rate and started to slow between 48-72 hours. This decrease was probably due to the fact that the cells were beginning to enter apoptosis since they

had been in the absence of foetal calf serum for a long period of time or that the mRNA expression was short-lived and was not being translated after 24 hours.

The stimulation with IL-1α, TNF-α and IFN-γ in combination (cytomix) yielded a more rapid and higher secretion of IL-8/CXCL8, due to an addition of the separate IL-1α and TNF-α effects. These results indicated that a combination of IL-1α and TNF-α, that induced high IL-8/CXCL8 mRNA expression was translated into high levels of secreted IL-8/CXCL8.

4.2.2 The secretion of IL-8/CXCL8 protein by the MKN45 cell line stimulated with IL-1α (10ng/ml) or TNF-α (100ng/ml) or 'cytomix' during a time course

Similar to the results obtained in the Northern analysis experiments and also similar to the results of the IL-8/CXCL8 ELISA obtained from the AGS cell line, the MKN45 cells did not secrete any IL-8/CXCL8 protein when unstimulated (Fig.25).

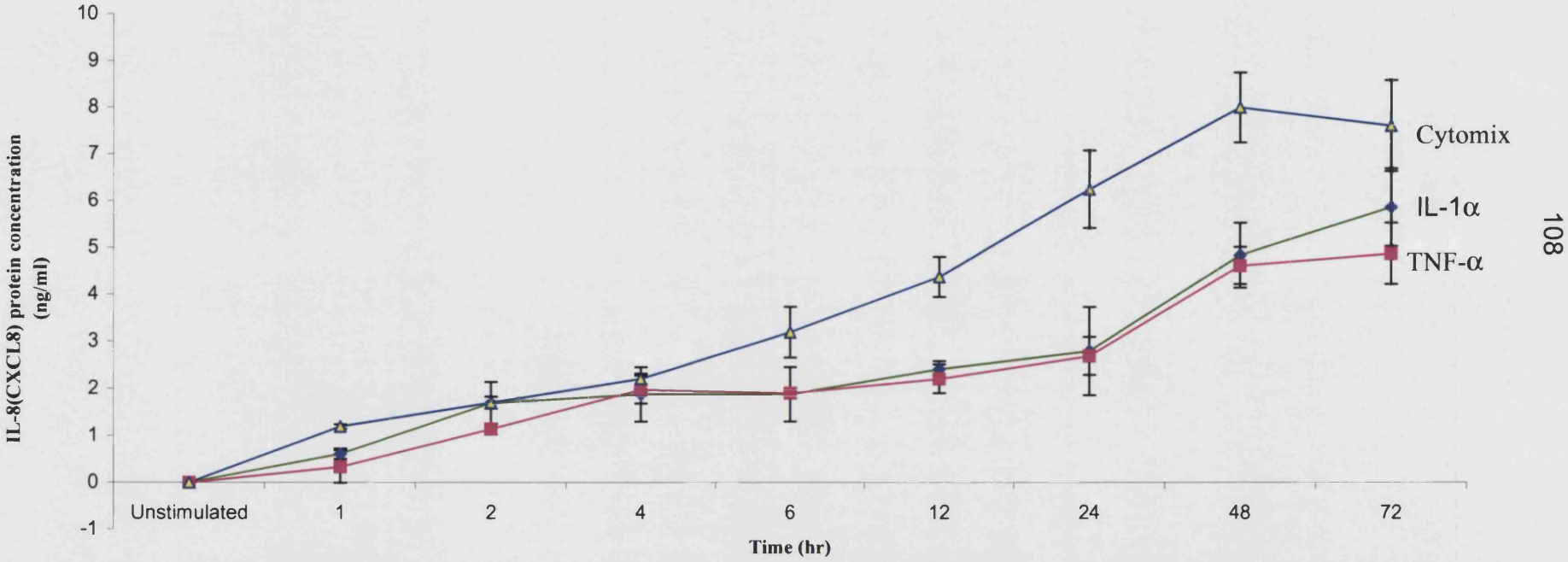
When the MKN45 cell line was stimulated with IL-1α(10ng/ml), low levels of IL-8/CXCL8 protein was secreted in a time-dependent manner. This suggests that IL-1α is a fairly weak stimulant in this cell line as it was also shown to induce very short-lived IL-8/CXCL8 mRNA expression. The MKN45 cell line exhibited a time dependent increase in the concentration of IL-8/CXCL8 protein secreted from 1.38 ± 0.13ng/ml at 1 hour to 3.64 ± 0.74ng/ml at 72 hours post-stimulation when it was stimulated with IL-1α (10ng/ml) (Fig.25).

Secreted IL-8/CXCL8 protein levels were higher when the MKN45 cells were stimulated with TNF-α compared to IL-1α. The levels of IL-8/CXCL8 did not increase after 48 hours stimulation with TNF-α since the MKN45 cells were starting to undergo apoptosis due to foetal bovine serum depletion. This could also be explained by the IL-8/CXCL8 mRNA levels which had decreased after a few hours.

When the 'cytomix' combination was used to stimulate the MKN45 cells, high IL-8/CXCL8 was secreted similar to levels seen when TNF-α was added alone.

Fig.24

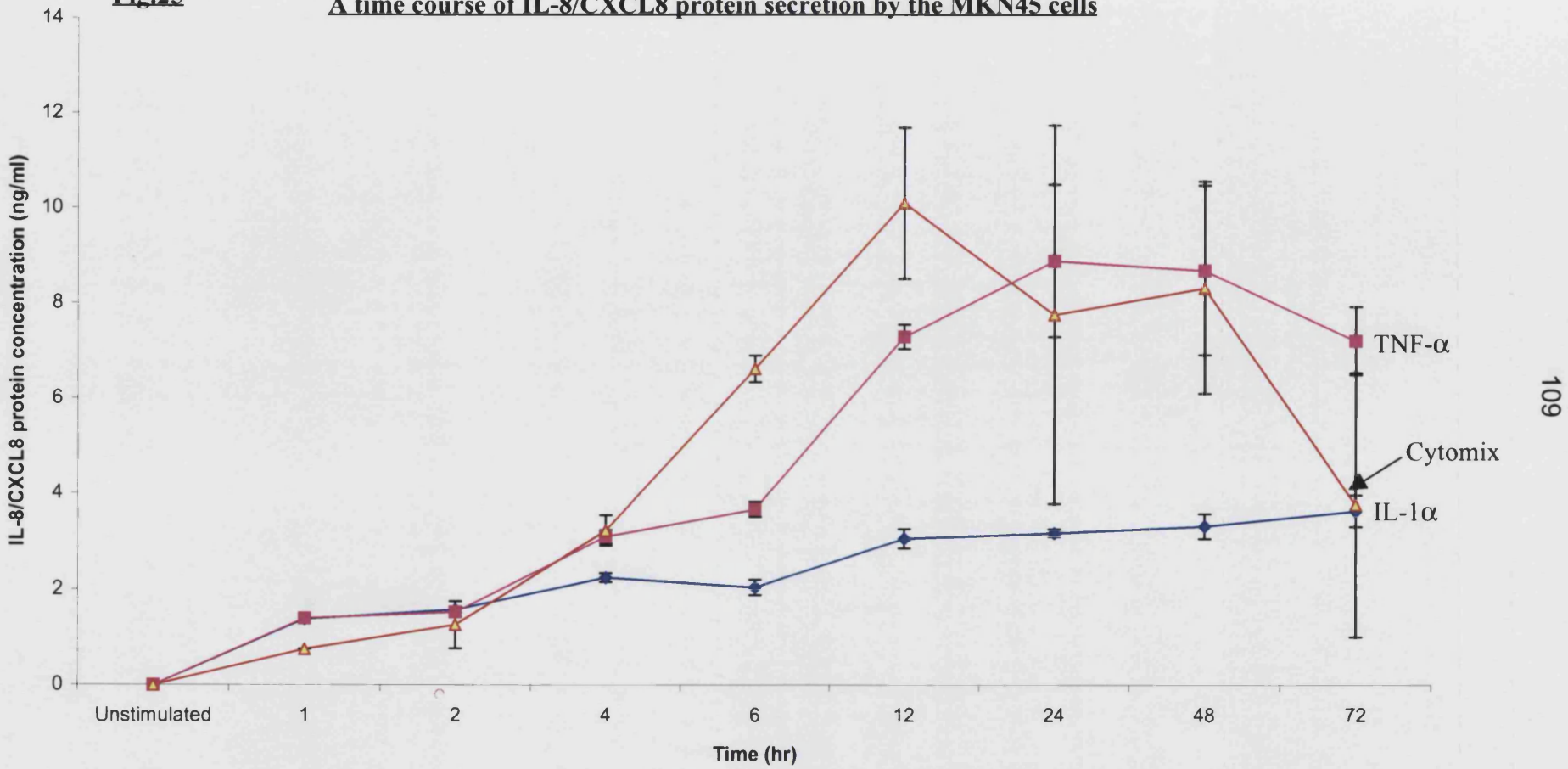
A time course of IL-8/CXCL8 protein secretion by the AGS cell line



The AGS cells were stimulated with IL-1α(10ng/ml) or TNF-α(100ng/ml) or 'cytomix' which is a combination of IL-1α(10ng/ml), TNF-α(100ng/ml) and IFN-γ(300U/ml) from 1 hr-72 hrs. Unstimulated cells were in medium only and the supernatants were removed from the unstimulated cells at the start of the experiment. At the specific times, the supernatants were collected, centrifuged to remove the cell debris and the IL-8/CXCL8 protein was analysed using an ELISA technique. Each point is the mean (+/-) SEM of three independent experiments.

Fig.25

A time course of IL-8/CXCL8 protein secretion by the MKN45 cells



The MKN45 cells were stimulated with IL-1α(10ng/ml), TNF-α(100ng/ml) or 'cytomix' which was a combination of IL-1α(10ng/ml), TNF-α(100ng/ml) and IFN-γ(300U/ml) from 1-72 hours. Unstimulated cells were in medium only, the supernatants were removed at the start of the experiment. At the specific times, the supernatants were collected, centrifuged to remove the cell debris and the IL-8/CXCL8 protein was analysed using an ELISA technique. Each point is the mean (+/-) SEM of three independent experiments.

4.2.3 The secretion of RANTES/CCL5 protein by the MKN45 cell line stimulated with TNF-α(100ng/ml) and IFN-γ(300U/ml) or 'cytomix'

As shown in the Northern analysis, the AGS cells did not express RANTES/CCL5 mRNA when stimulated with IL-1α, TNF-α or IFN-γ. Therefore, as expected, RANTES/CCL5 protein was also not detected in secretions of this cell line (results not shown).

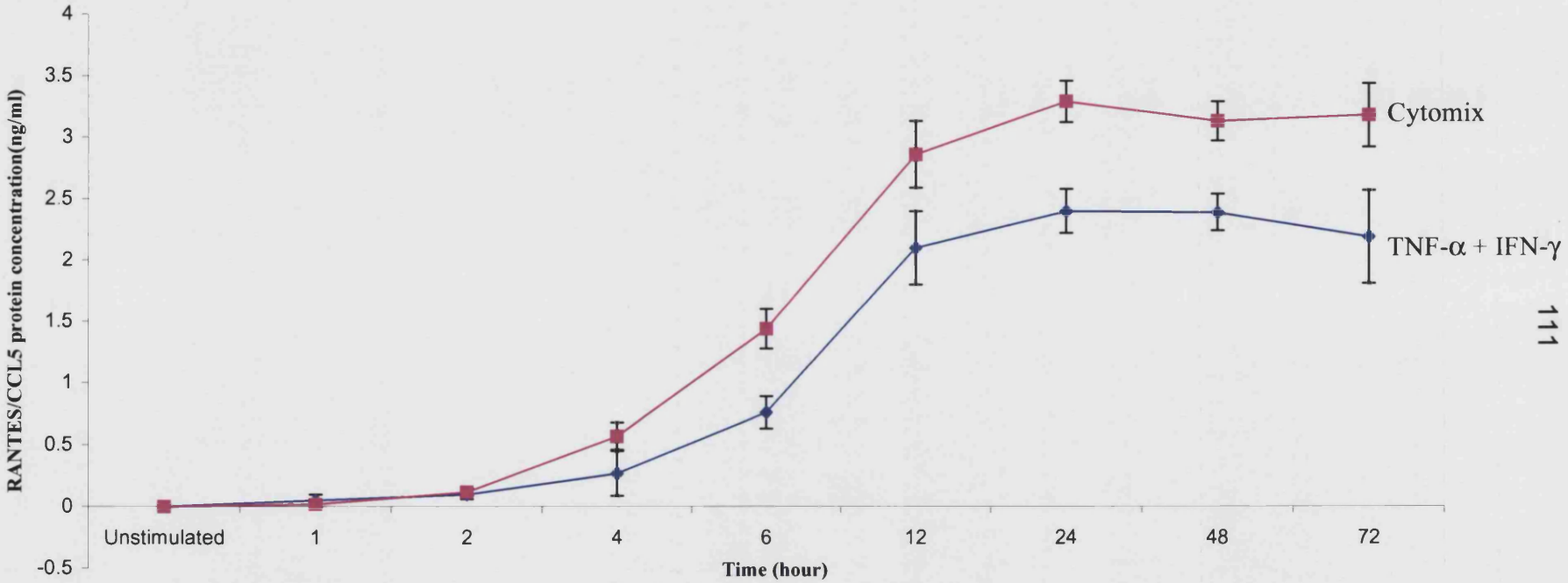
Cytokines added alone to the MKN45 cells did not induce RANTES/CCL5 protein (results not shown). Stimulation with TNF-α and IFN-γ or cytomix induced a similar time-dependent secretion of RANTES/CCL5 protein (Fig.26). This indicated that IL-1α was not important in inducing RANTES/CCL5 expression. The concentration of RANTES/CCL5 protein secreted in response to 1 hour stimulation with TNF-α and IFN-γ was 0.05 ± 0.1ng/ml and at 72 hours was 2.19 ± 0.76ng/ml. 'Cytomix' stimulation yielded RANTES/CCL5 protein levels of 0.02 ± 0.05ng/ml at 1 hour increasing to 3.29 ± 0.33ng/ml at 72 hours post-stimulation. RANTES/CCL5 protein expression was not detected very early (1-2 hours) since the RANTES/CCL5 mRNA expression was detected later (6-12 hours).

4.3 THE EFFECTS OF ANTI-INFLAMMATORY CYTOKINES ON THE EXPRESSION OF CHEMOKINES

Having investigated IL-8/CXCL8 and RANTES/CCL5 expression in response to stimulation with pro-inflammatory cytokines IL-1α, TNF-α and IFN-γ, this study was extended to investigate the modulatory effects of the cytokines IL-4 and IL-13 produced by Th2 cells. These cytokines are known to have anti-inflammatory effects in some situations. IL-4 and IL-13 have previously been shown to be inhibitory in HT-29 colonic epithelial cell RANTES/CCL5 protein expression (Kolios et al., 1999) but in other cells, these cytokines can be stimulatory. In smooth muscle cells, IL-13 increased IL-1 induced IL-8/CXCL8 expression (Jordan et al., 1997). The experiments in this next section were carried out to determine the effects of these cytokines on gastric epithelial cells.

Fig.26

A time course of RANTES/CCL5 protein by the MKN45 cell line



MKN45 cells were stimulated with TNF-α(100ng/ml) and IFN-γ(300U/ml) or 'cytomix' which is a combination of IL-1α(10ng/ml), TNF-α(100ng/ml) and IFN-γ (300U/ml) from 1 hr-72 hrs. Unstimulated cells were in medium only and the supernatants were removed at the start of the experiment. At the specific times, the supernatants were collected, centrifuged to remove the cell debris and the RANTES/CCL5 protein was studied using an ELISA technique. Each point is the mean (+/-) SEM of three independent experiments.

4.3.1 The effect of IL-4 and IL-13 on the expression of induced IL-8/CXCL8 protein in AGS cells

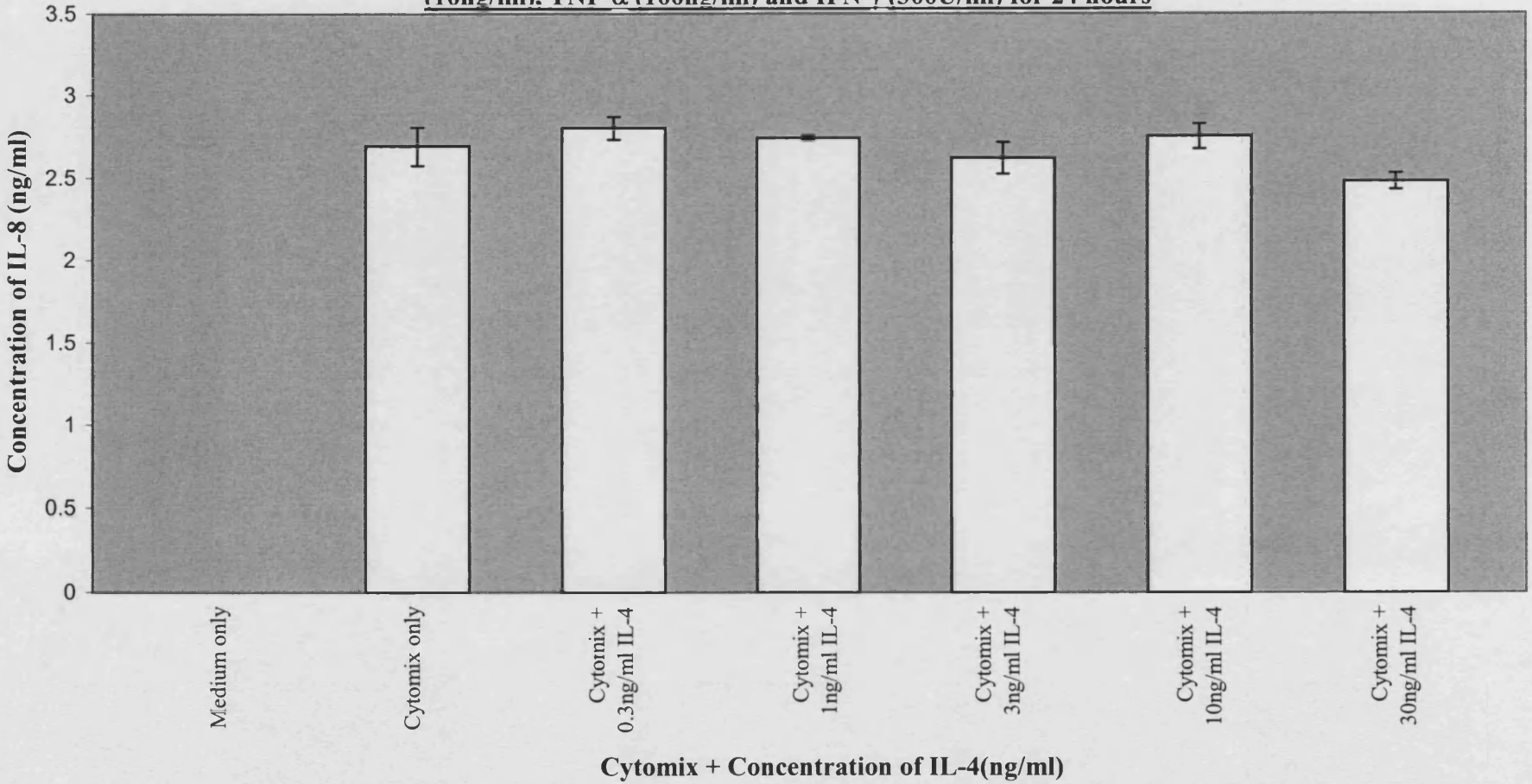
AGS cells were incubated overnight in serum free medium prior to the experiment. IL-4 or IL-13 was added to the cells and incubated for 1 hour at 37°C, then cytomix was added and incubation continued for 24 hours. The medium was removed and IL-8/CXCL8 was measured by ELISA. Unstimulated AGS cells did not secrete IL-8 protein. Cells stimulated for 24 hours with 'cytomix' secreted IL-8/CXCL8 protein as shown in Fig.27 and Fig.28 when they were pre-stimulated with IL-4 and IL-13 respectively. The effects of IL-4 and IL-13 on the secretion of IL-8/CXCL8 protein were studied. All concentrations of IL-4 and IL-13 (0.3-30ng/ml) did not have any significant effect on IL-8/CXCL8 production. The ANOVA and Dunnett's tests carried out on this data indicated that in this cell line, IL-4 and IL-13 did not act as stimulating or inhibitory cytokines on IL-8/CXCL8 production ($P>0.05$).

4.3.2 The effect of IL-4 on the expression of induced IL-8/CXCL8 protein and mRNA by the MKN45 cell line

The MKN45 cells were stimulated with cytomix in the presence of IL-4 as described for the AGS cells (section 4.3.1). The ELISA results demonstrated that there was no significant effect of IL-4 (0.3-30ng/ml) on IL-8/CXCL8 protein secretion in the MKN45 cell line (Fig.29) as shown by the ANOVA and Dunnett's statistical tests. This was similar to the results obtained with the AGS cells. The expression of IL-8/CXCL8 mRNA in this experiment was also investigated by Northern analysis after a 24 hour stimulation with IL-4 and cytomix (Fig.30). Similar to the results obtained via the ELISA experiment, the Northern experiment did not show that there was any change in the relative concentration of IL-8/CXCL8 mRNA with increasing concentrations of IL-4 used.

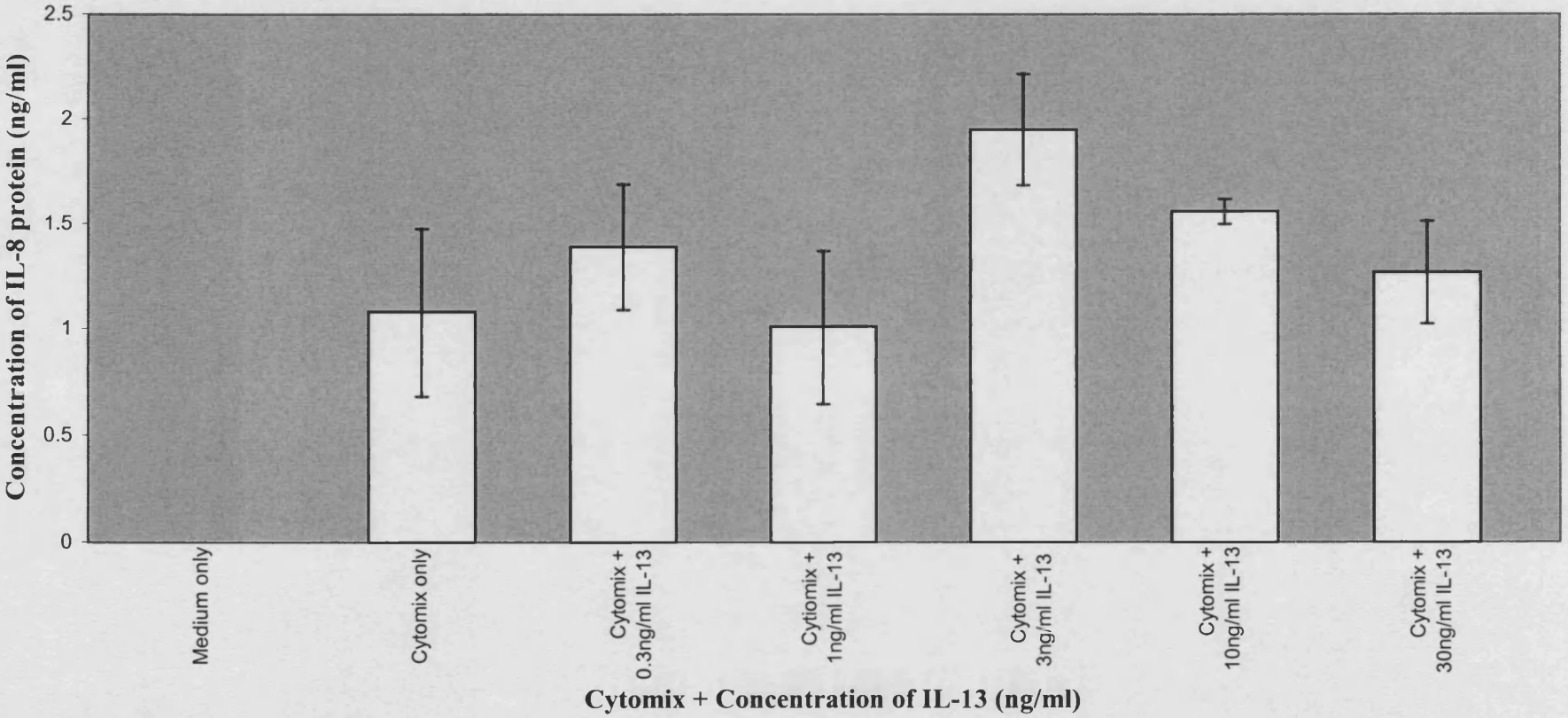
Fig.27

The secretion of IL-8/CXCL8 protein by AGS cells pre-treated with IL-4 (1 hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 24 hours



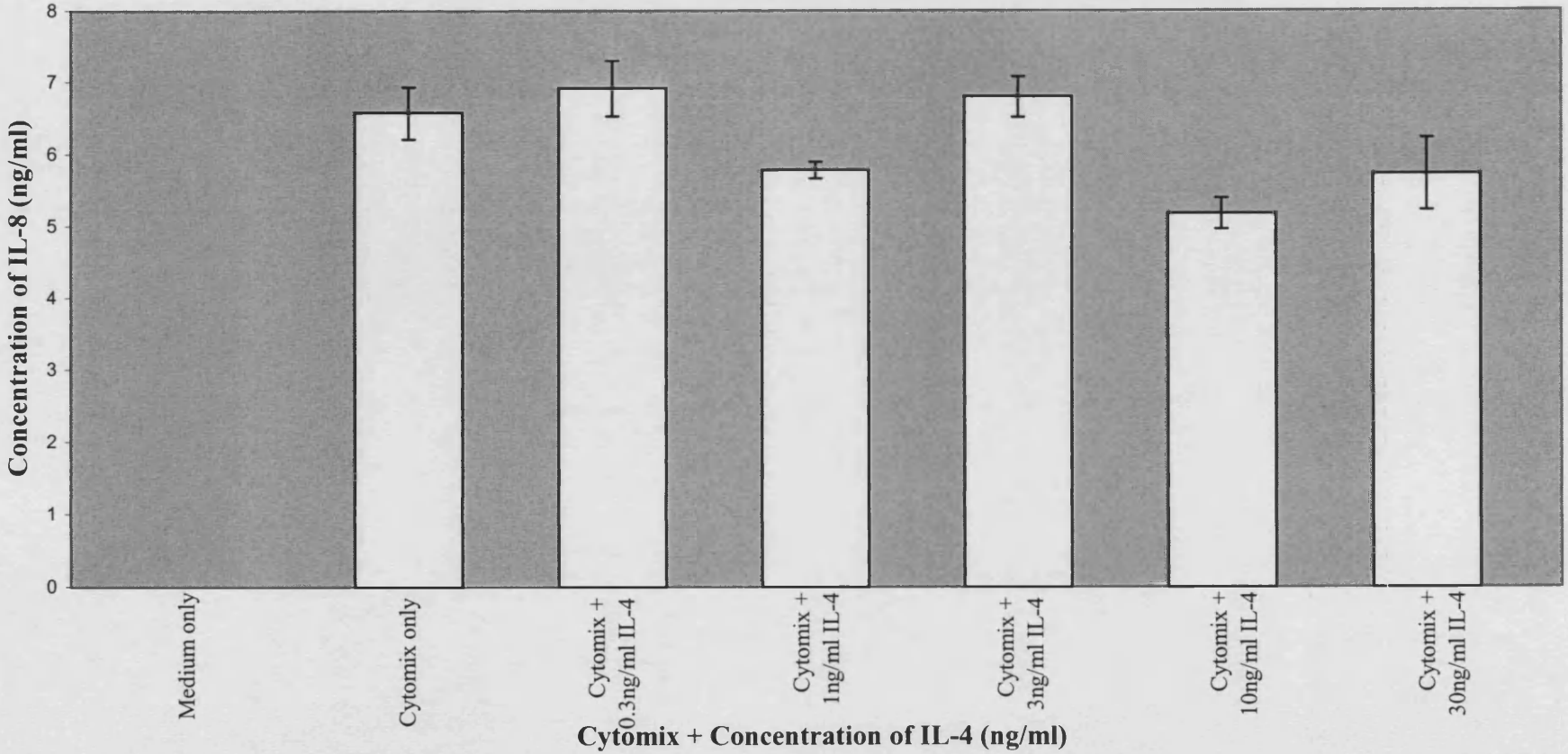
The effect of pre-treating the AGS cells with IL-4 for an hour before stimulating them with 'cytomix' for 24 hours on the expression of IL-8/CXCL8 protein. Each bar is the mean (+/-) SEM of three independent experiments.

Fig.28 The secretion of IL-8/CXCL8 protein by AGS cells pre-treated with IL-13 (1 hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 24 hours



The effect of pre-stimulating the AGS cells with IL-13 for an hour before stimulating them with 'cytomix' for 24 hours on the expression of IL-8/CXCL8 protein. Each bar is the mean (+/-) SEM of three independent experiments.

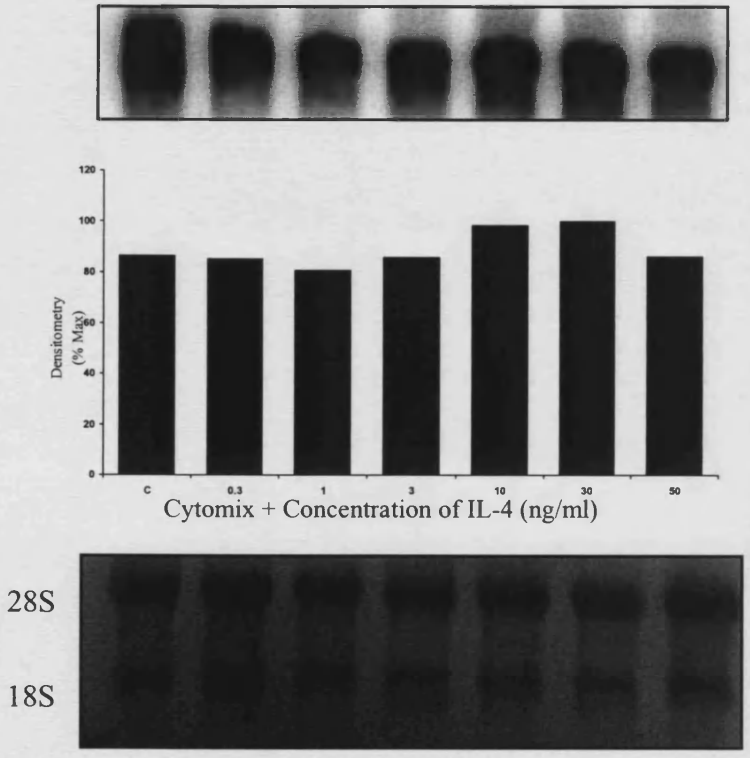
Fig.29 **The expression of IL-8/CXCL8 protein by MKN45 cells pre-stimulated with IL-4 (1 hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 24 hours**



The effect of pre-stimulating the MKN45 cells with IL-4 for an hour before stimulating them with 'cytomix' for 24 hours on the expression of IL-8/CXCL8 protein. Each bar is the mean (+/-) SEM of three independent experiments.

Fig. 30

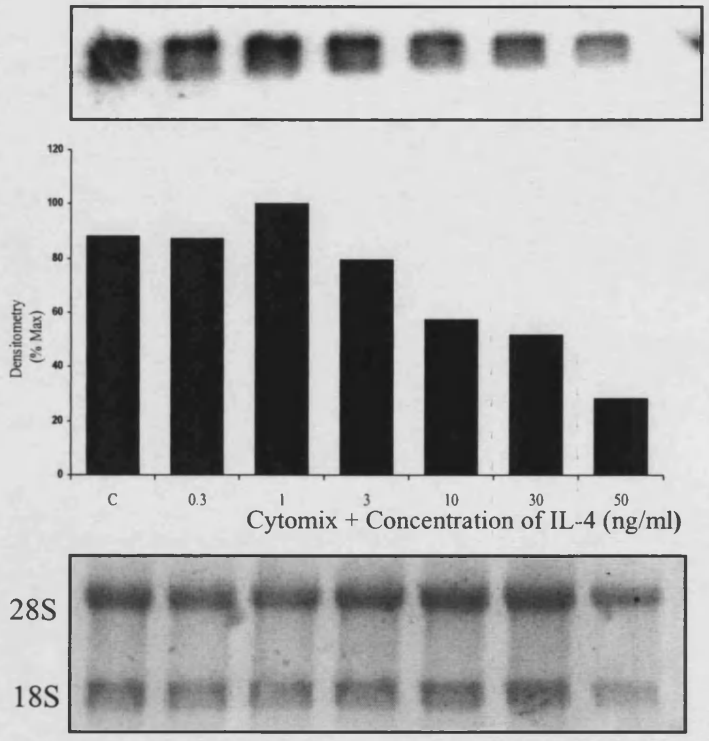
The expression of IL-8 mRNA by MKN45 cells pre-stimulated with IL-4 (for an hour) and stimulated with IL-1α (10ng/ml) ,TNF-1α (100ng/ml) and IFN-γ (300U/ml) for 24 hours



MKN45 cells were pre-stimulated with different concentrations of IL-4 for an hour and stimulated with a combination of IL-1α, TNF-α and IFN-γ for 24 hours. 'C' represents cells stimulated with IL-1α, TNF-α and IFN-γ only. After 24 hours, the cells were lysed into 'RNAzol B'. RNA was extracted and run onto a 1% agarose/formaldehyde gel. IL-8 mRNA was detected by Northern analysis (top panel). Relative concentrations of IL-8 mRNA were measured by scanning densitometry (middle panel). The bottom panel shows the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.31

The expression of RANTES mRNA by MKN45 cells pre-stimulated with IL-4 (for an hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 48 hours



MKN45 cells were pre-stimulated with different concentrations of IL-4 for an hour and stimulated with a combination of IL-1α, TNF-α and IFN-γ for 48 hours. 'C' represents cells stimulated with IL-1α, TNF-α and IFN-γ only. After 48 hours, the cells were lysed into 'RNAzol B'. RNA was extracted and run onto a 1% agarose/ formaldehyde gel. RANTES mRNA was detected by Northern analysis (top panel). Relative concentrations of RANTES mRNA were measured by scanning densitometry (middle panel). The bottom panel shows the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

4.3.3 The effect of IL-4 on the expression of induced RANTES/CCL5 mRNA and protein by MKN45 cell line

RANTES/CCL5 mRNA and protein were measured in the MKN45 cells which were pre-treated for 1 hour with IL-4 (0.3-30ng/ml) and stimulated with cytomix for 48 hours. The ELISA results demonstrated that unstimulated MKN45 cells did not secrete any RANTES/CCL5 protein. RANTES/CCL5 was secreted after 48 hours stimulation with cytomix (Fig.32). The highest concentrations of IL-4 used, 10ng/ml and 30ng/ml (together with cytomix) caused an approximately 31% and 25% decrease in RANTES/CCL5 secretion respectively compared to cytomix only control. Dunnett's test indicated that these concentrations of IL-4 significantly ($P<0.05$) inhibited RANTES/CCL5 secretion by MKN45 cells compared to cytomix only control.

The Northern analysis shown in Fig.31 indicated diminished expression of cytomix-induced RANTES/CCL5 mRNA with higher concentrations of IL-4 which were 10, 30 and 50ng/ml.

4.3.4 The effect of IL-13 on the expression of IL-8/CXCL8 mRNA by MKN45 cells

As shown in Fig.30, IL-4 did not modulate cytomix-induced IL-8/CXCL8 mRNA expression in MKN45 cells, similarly the Th2 cytokine, IL-13 at concentrations 0.3-30ng/ml did not modulate IL-8/CXCL8 mRNA expression (Fig.33).

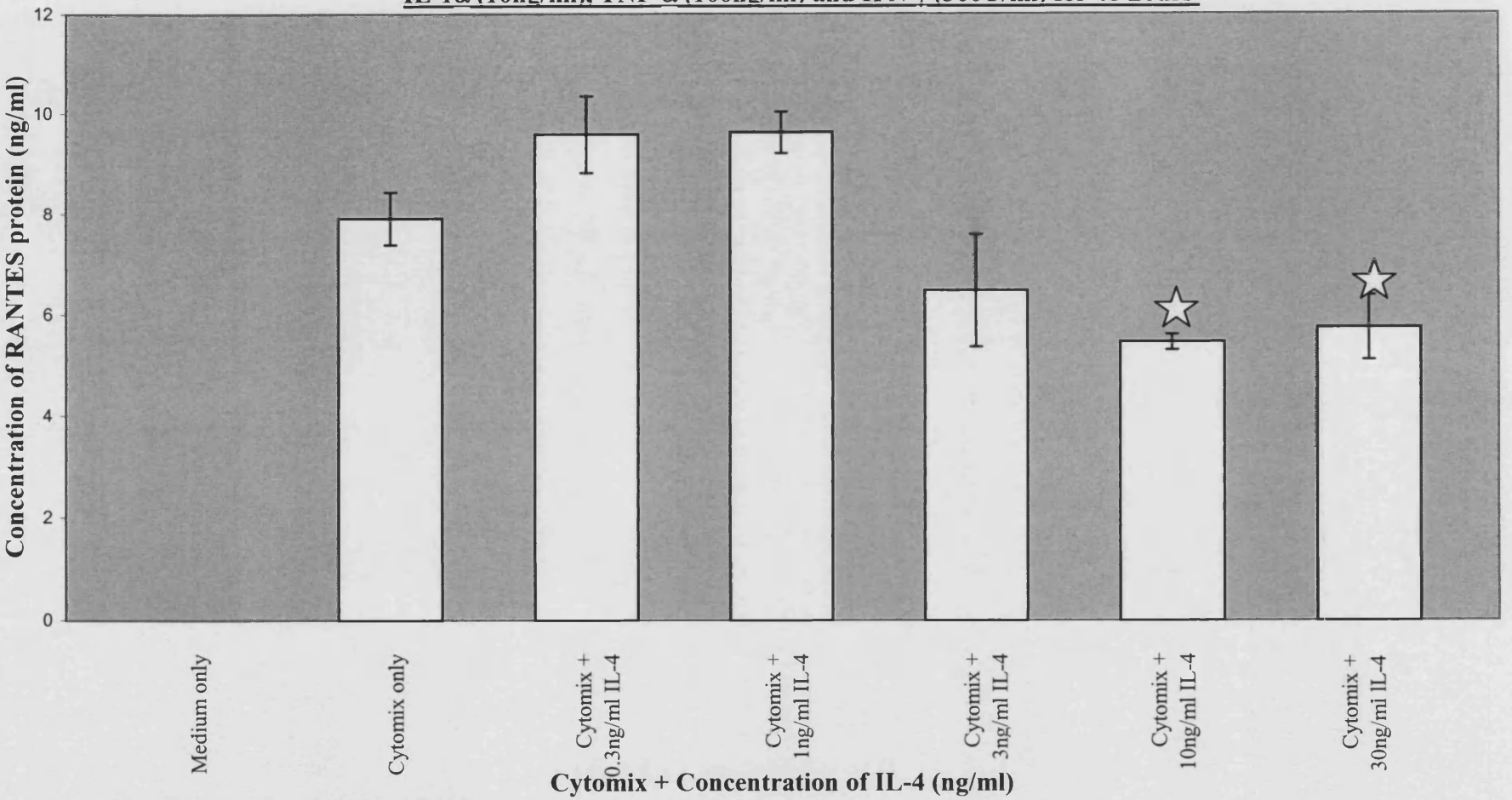
4.3.5 The effect of IL-13 on the expression of RANTES/CCL5 protein by MKN45 cell line

RANTES/CCL5 protein were measured in MKN45 cells pre-treated for 1 hour with IL-13 (0.3-30ng/ml) and stimulated with cytomix for 48 hours. Unstimulated MKN45 cells did not express any RANTES/CCL5 protein (Fig.35). RANTES/CCL5 protein was secreted when the MKN45 cells were stimulated with cytomix (7.57 ± 0.61ng/ml). Significance was assessed by ANOVA and Dunnett's test ($P<0.05$) (Fig.35). Similar to the effect of IL-4, IL-13 was found to inhibit the expression of

RANTES/CCL5 protein. The highest concentrations of IL-13 used (10 and 30ng/ml), together with cytomix caused a 33.3% and 60% decrease in RANTES/CCL5 secretion compared to cytomix only control. Dunnett's test indicated that these concentrations of IL-13 significantly ($P<0.05$) inhibited RANTES/CCL5 secretion compared to cytomix only control.

The effect of IL-13 on RANTES/CCL5 mRNA expression was investigated by Northern analysis. IL-13(30ng/ml) caused a decrease in cytomix stimulated RANTES/CCL5 mRNA (Fig.34). This is in agreement with the results obtained by ELISA.

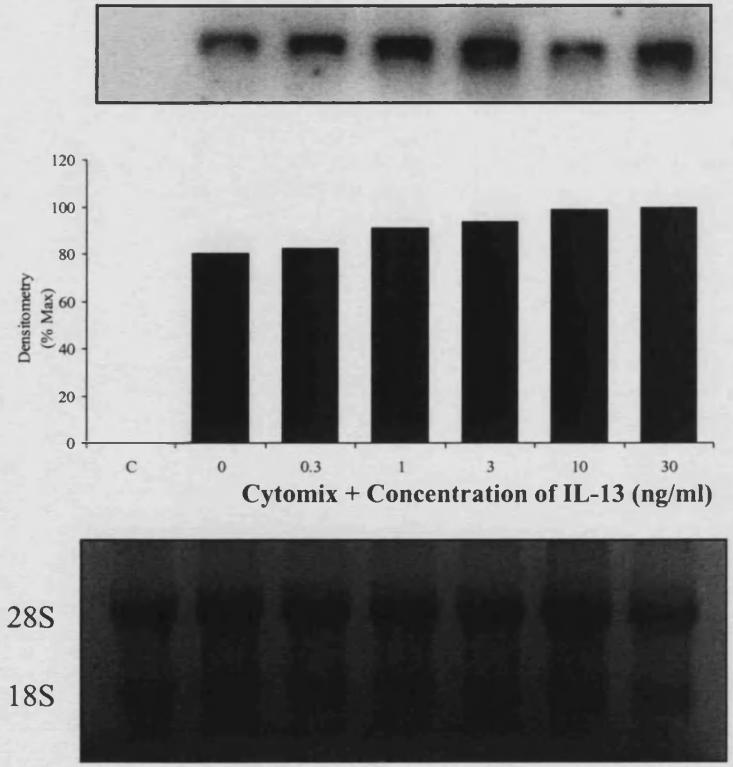
Fig.32 **The secretion of RANTES/CCL5 protein by MKN45 cells pre-stimulated with IL-4 (1 hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 48 hours**



The effect of pre-stimulating the MKN45 cells with IL-4 for an hour and stimulating them with 'cytomix' for 48 hours on the expression of RANTES protein. Each bar is the mean (+/-) SEM of three independent experiments. The significance of using IL-4 with cytomix compared to cytomix alone on the secretion of RANTES/CCL5 protein was assessed by ANOVA and Dunnett's test ($P<0.05$).

Fig.33

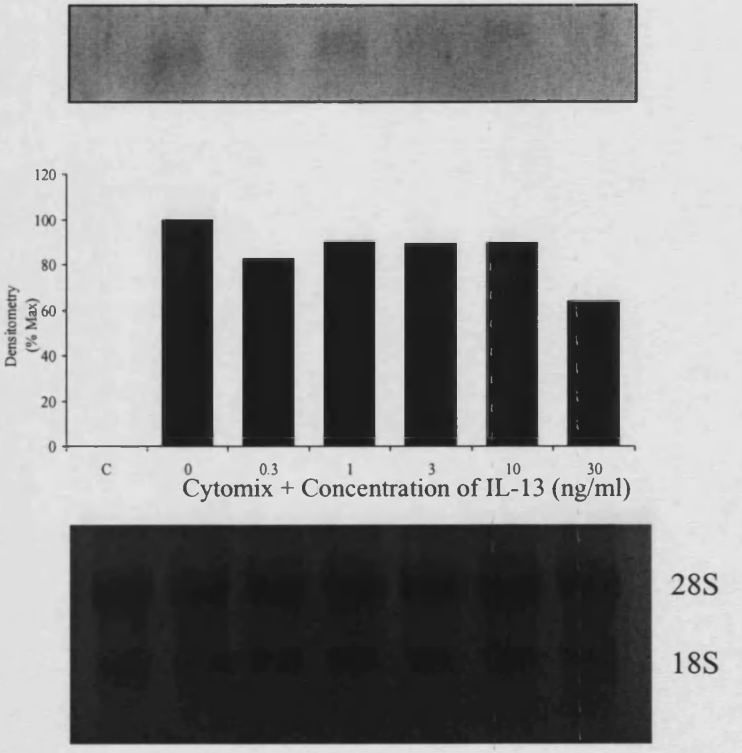
The expression of IL-8/CXCL8 mRNA by MKN45 cells pre-stimulated with different concentrations of IL-13 (for an hour) and stimulated with IL-1α (10 ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 24 hours



MKN45 cells were pre-stimulated with different concentrations of IL-13 for an hour. 'C' represents unstimulated control cells. After stimulation with IL-13, the cells were stimulated with IL-1α, TNF–α and IFN-γ for 24 hours before the cells were lysed in 'RNAzol B'. RNA was extracted and run onto a 1% agarose/formaldehyde gel. IL-8 mRNA was detected via Northern analysis (top panel). Relative concentrations of IL-8 mRNA were detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

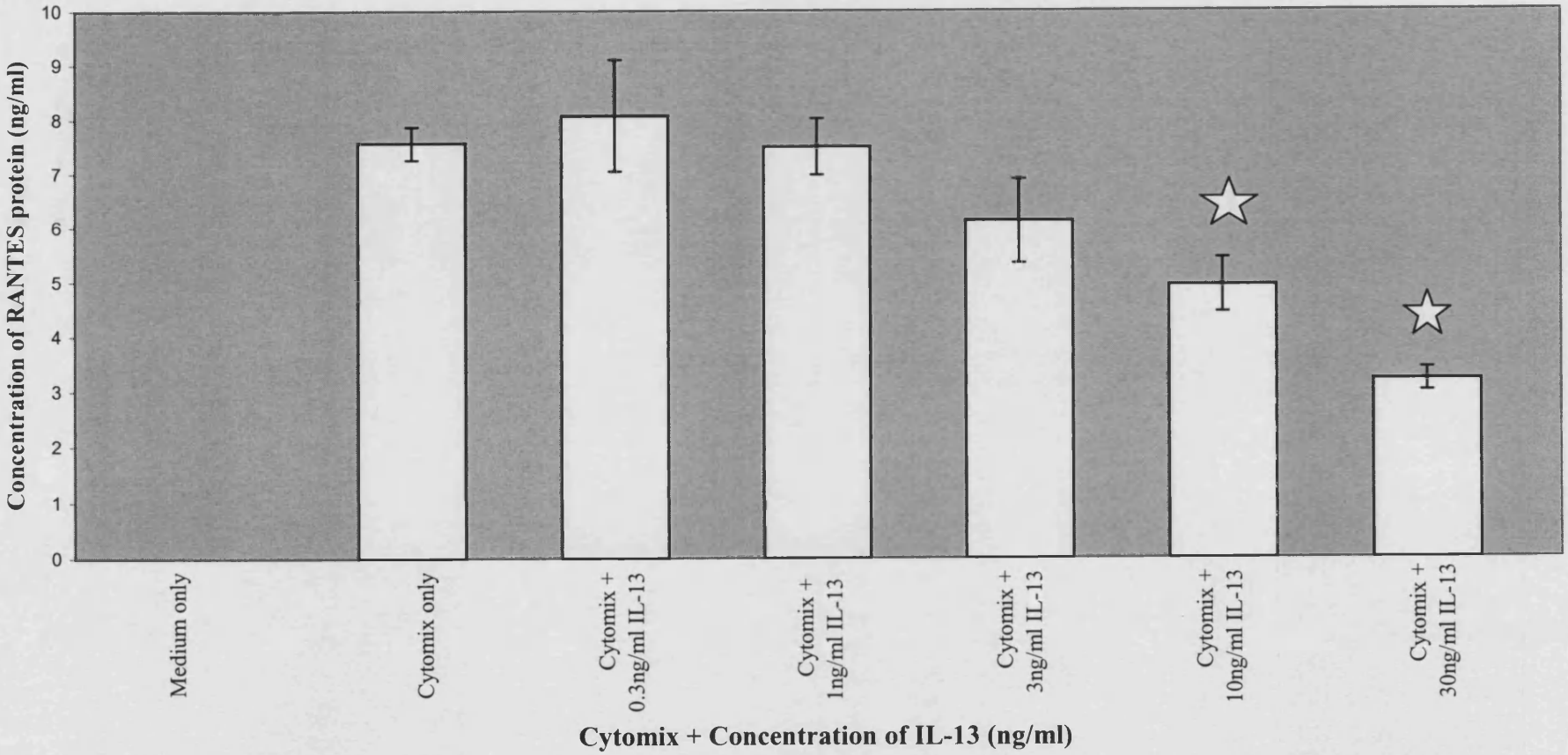
Fig.34

The expression of RANTES/CCL5 mRNA by MKN45 cells pre-stimulated with different concentrations of IL-13 (for an hour) and stimulated with IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) for 48 hours



MKN45 cells were pre-stimulated with different concentrations of IL-13 for an hour. 'C' represents unstimulated control cells. After stimulation with IL-13, the cells were stimulated with IL-1α, TNF-α and IFN-γ for 48 hours before the cells were lysed in 'RNAzol B'. RANTES mRNA was detected by Northern analysis (top panel). RNA was extracted and run onto a agarose/formaldehyde gel. Relative concentrations of IL-8 mRNA were detected by scanning densitometry (middle panel). The bottom panel is the ethidium bromide-stained 18S and 28S bands indicating equal loading of the lanes. Representative of three independent experiments.

Fig.35 **The secretion of RANTES/CCL5 protein by MKN45 cell pre-stimulated with IL-13 (1 hour) and stimulated with IL-1α (10ng/ml), TNF-α(100ng/ml) and IFN-γ(300U/ml) for 48 hours**



The effect of pre-stimulating the MKN45 cells with IL-13 for an hour and stimulating them with 'cytomix' for 48 hours on the expression of RANTES protein. Each bar is the mean (+/-) SEM of three independent experiments. The significance of using IL-13 together with cytomix compared to using cytomix alone on the RANTES/CCL5 protein secretion was assessed by ANOVA and Dunnett's test ($P<0.05$).

4.4 THE EXPRESSION OF A RANGE OF 'CC' AND 'CXC' CHEMOKINE mRNA IN AGS AND MKN45 CELLS DETECTED BY RT-PCR

The expression of the prototype chemokines from the 'CXC' and 'CC' families, IL-8/CXCL8 and RANTES/CCL5 were initially investigated in detail by Northern analysis. This study provided information about time courses of expression of IL-8/CXCL8 and RANTES/CCL5 and the combination of cytokines required for gastric epithelial cell activation. This information was used to generate suitable MKN45 and AGS samples which were then analysed for expression of a range of novel 'CXC' and 'CC' chemokines using the sensitive technique of PCR.

For each cell line, 10 samples were used per experiment, which were the unstimulated AGS/MKN45 cell line serum-starved overnight, the AGS/MKN45 cell line stimulated with 'cytomix' for 1, 3 and 12 hours and a positive control. Negative controls included RT minus samples for each cell line sample and a water only control to detect any contamination in reagents. Positive control samples were used to validate that the primer pairs could detect the appropriate mRNA.

Cytomix stimulated HT-29 cells were used as a positive control for PCR primers against IL-8/CXCL8, MIG/CXCL9, IP-10/CXCL10 and MCP-1/CCL2. White blood cells stimulated with 50ng/ml PHA were used as positive controls for BCA-1/CXCL13, MIP-1α/CCL3, TARC/CCL17 and LARC/CCL20. U937 cells stimulated with 50ng/ml PMA was the positive control for RANTES/CCL5 and MCP-3/CCL7. Fibroblasts were used as a positive control for SDF-1α/CXCL12. 'Cytomix' or a combination of IL-1α (3ng/ml), TNF-α (30ng/ml) and IFN-γ (100U/ml) were used as a stimuli for the AGS and MKN45 cell lines so that maximal expression of chemokines could be observed. These results were obtained from three separate experiments. On all gels, a 100 base pair DNA ladder was used and the lanes were numbered from left to right. The results obtained from the PCR analysis are summarised in Table 5.

Table 5. Chemokine expression by the AGS and MKN45 gastric epithelial cell lines as derived from RT-PCR

	AGS cell line					MKN45 cell line				
Chemokine	**Control (un-stimulated)**	**Cytomix (1hr)**	**Cytomix (3hrs)**	**Cytomix (12 hrs)**	**Positive control**	**Control (un-stimulated)**	**Cytomix (1 hr)**	**Cytomix (3 hrs)**	**Cytomix (12 hrs)**	**Positive control**
CXC										
IL-8 (CXCL8)	+	+	+++	+	++	++	+++	++	+++	+++
MIG (CXCL9)	+	++	++	+++	+++	+	+++	+++	+++	+++
IP-10 (CXCL10)	-	-	-	-	++	-	++	+++	++	+++
SDF-1α (CXCL12)	-	-	-	-	++	-	-	-	-	++
BCA-1 (CXCL13)	-	-	-	-	+++	-	-	-	-	+++
CC										
MCP-1 (CCL2)	-	+	+	-	++	-	+	++	+	+++
MIP-1α (CCL3)	-	-	-	-	+++	-	-	-	-	+++
RANTES (CCL5)	-	-	-	-	+++	-	-	-	++	+++
MCP-3 (CCL7)	-	-	+	+	++	-	+	+	+	++
TARC (CCL17)	-	-	-	-	++	-	-	-	-	++
LARC (CCL20)	+	+++	+++	+++	+++	+++	+++	+++	+++	+++

Results shown on gels

+++ = very strong signal; ++ = strong signal; + = weak signal; - = not detected

AGS cells

The AGS cell line was shown to express barely detectable constitutive levels of the 'CXC' chemokines IL-8/CXCL8 (Fig.36) and MIG/CXCL9 (Fig.37) mRNA. These low levels were able to be detected probably due to the sensitivity of the RT-PCR technique compared to the Northern analysis which was not able to detect the constitutive levels of IL-8/CXCL8 mRNA. IL-8/CXCL8 mRNA expression was stimulated by cytomix to a maximum by 3 hours and had decreased by 12 hours and this supports the results detected by Northern analysis. When the AGS cell line was stimulated with 'cytomix', the expression of MIG/CXCL9 was stimulated higher than the basal levels with prolonged high expression between 1-12 hours. Expression of other members of the 'CXC' family, IP-10/CXCL10, SDF-1α/CXCL12 and BCA-1/CXCL13 mRNA expression were also analysed by PCR, no expression was detected in 'cytomix' stimulated or unstimulated AGS cells (see Table 5).

The 'CC' chemokine MCP-1/CCL2 (Fig.39) mRNA was expressed very weakly (barely detectable) in 'cytomix' stimulated cells, from 1-3 hours while MCP-3/CCL7 (Fig.40) was also detected faintly at 3-12 hours. In contrast, the 'CC' chemokine LARC/CCL20 was detected at high levels in cells stimulated between 1-12 hours with cytomix, with the highest level of expression at 12 hours. LARC/CCL20 was also expressed at low levels in unstimulated cells (Fig.38). Fig.41 demonstrates a representative β-actin blot for the samples, indicating equal loading of all samples.

Expression of other members of the 'CC' chemokine family were also investigated by RT-PCR. mRNA expression in stimulated or unstimulated cells were not detected for MIP-1α/CCL3, RANTES/CCL5 and TARC/CCL17 (see Table 5).

Chemokine expression by AGS cells stimulated with cytomix

On all gels, a 100 bp DNA ladder was used and lanes are numbered from left to right.

1 2 3 4 5 6 7 8 9 10

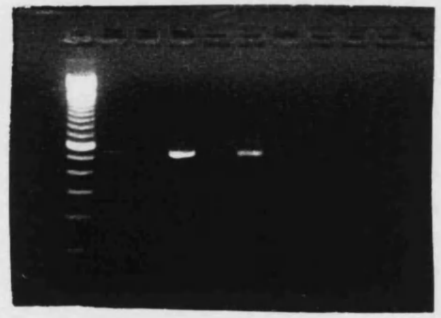


Fig.36 PCR analysis of IL-8/CXCL8 mRNA expression in AGS cells (562 bp)

1= Unstimulated AGS cells (very faint)
2= AGS cells stimulated with cytomix for 1 hour (very faint)
3= AGS cells stimulated with cytomix for 3 hours
4= AGS cells stimulated with cytomix for 12 hours (very faint)
5= Positive control (HT-29 cells stimulated with cytomix)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

1 2 3 4 5 6 7 8 9 10

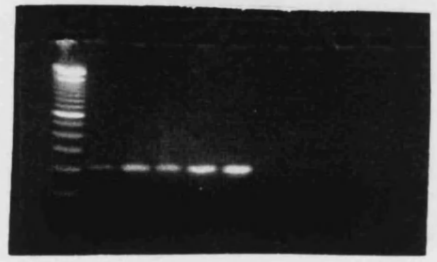


Fig.37 PCR analysis of MIG/CXCL9 expression in AGS cells (214 bp)

1= Unstimulated AGS cells (very faint)
2= AGS cells stimulated with cytomix for 1 hour
3= AGS cells stimulated with cytomix for 3 hours
4= AGS cells stimulated with cytomix for 12 hours
5= Positive control (HT29 stimulated with cytomix)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

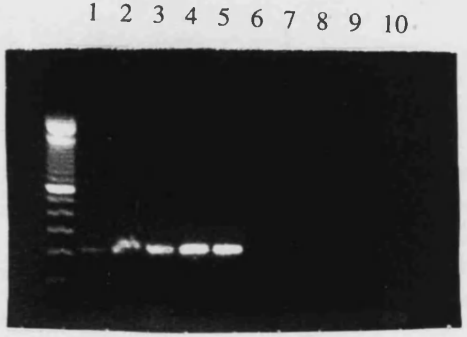


Fig.38 PCR analysis of LARC/CCL20 mRNA expression in AGS cells (210 bp)

1= Unstimulated AGS cells (very faint)
2= AGS cells stimulated with cytomix for 1 hour
3= AGS cells stimulated with cytomix for 3 hours
4= AGS cells stimulated with cytomix for 12 hours
5= Positive control (white blood cells stimulated with PHA)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is reprsentative of three independent experiments

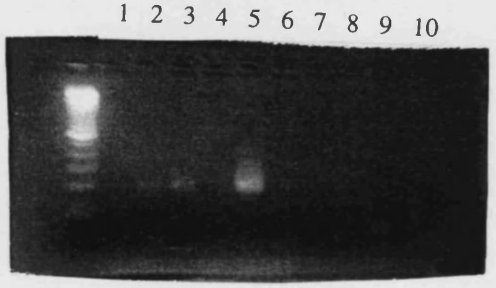


Fig.39 PCR analysis of MCP-1/CCL2 mRNA expression in AGS cells (177 bp)

1= Unstimulated AGS cells
2= AGS cells stimulated with cytomix for 1 hour (very faint)
3= AGS cells stimulated with cytomix for 3 hours (very faint)
4= AGS cells stimulated with cytomix for 12 hours
5= Positive control (HT29 stimulated with cytomix)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

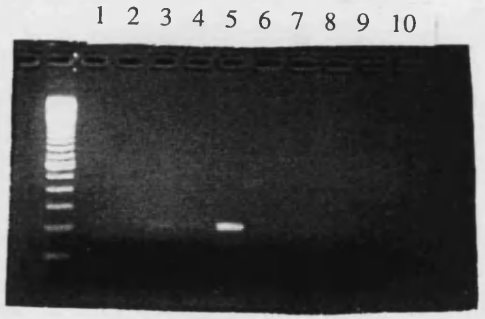


Fig.40 PCR analysis of MCP-3/CCL7 mRNA expression in AGS cells (216 bp)

1= Unstimulated AGS cells
2= AGS cells stimulated with cytomix for 1 hour
3= AGS cells stimulated with cytomix for 3 hours (very faint)
4= AGS cells stimulated with cytomix for 12 hours (very faint)
5= Positive control (U937 stimulated with PMA)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

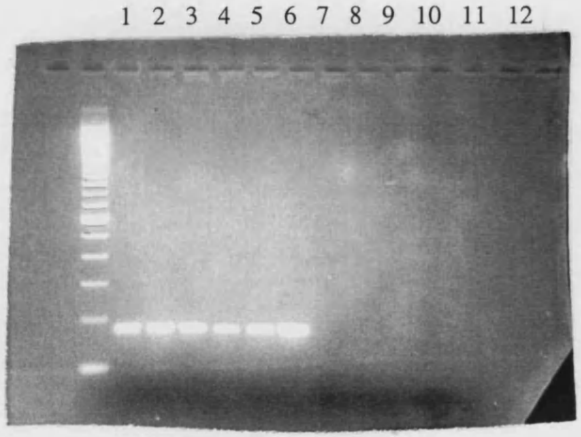


Fig. 41 PCR analysis of β-actin mRNA expression in AGS cells (176 bp)

1= Unstimulated AGS cells
2= AGS cells stimulated with cytomix for 1 hour
3= AGS cells stimulated with cytomix for 3 hours
4= AGS cells stimulated with cytomix for 6 hours
5 =AGS cells stimulated with cytomix for 12 hours
6= Positive control (HT29 stimulated with cytomix)
7-11= samples of 1-4 minus reverse transcriptase
12= water

This gel is representative of three independent experiments

MKN45 cells

The MKN45 cell line has been shown by RT-PCR to express the 'CXC' chemokines IL-8/CXCL8 (Fig.42). MKN45 cells expressed low levels of IL-8/CXCL8 mRNA constitutively, expression was increased from 1-12 hours stimulation with cytomix, with a peak at 1 hour, as seen in the Northern analysis experiment. The constitutive expression of IL-8/CXCL8 mRNA was detected by the RT-PCR experiment but not the Northern analysis experiment probably because RT-PCR is a more sensitive technique compared to the Northern analysis and is able to detect small quantities of the IL-8/CXCL8 mRNA. MIG/CXCL9 (Fig.43) mRNA was also expressed at low levels constitutively in MKN45 cells. MIG/CXCL9 mRNA was expressed very strongly between 1 and 12 hours (as was detected in the AGS cells). IP-10/CXCL10 peak expression occurred in MKN45 cells at 3 hours post-stimulation (Fig.44). The chemokines which were not detected in the unstimulated or cytomix stimulated AGS and MKN45 cell lines were SDF-1α/CXCL12 and BCA-1/CXCL13 (results listed in Table 5).

LARC/CCL20 (Fig.45) was expressed in MKN45 cells at high levels constitutively which is in contrast to the constitutive expression detected in AGS cells. In the presence of cytomix, LARC/CCL20 mRNA expression in MKN45 cells remained high from 1-12 hours. MCP-1/CCL2 (Fig.46) mRNA was expressed at high levels while MCP-3/CCL7 (Fig.47) mRNA was expressed at low levels in the blot.

The 'CC' chemokines MIP-1α/CCL3 and TARC/CCL17 were not detected (Table 5). This result was similar to that obtained for the AGS cell line. RANTES/CCL5 (Fig.48) mRNA expression was similar to that detected by MKN45 cells in the Northern analysis experiments with a late peak expression at 12 hours post-stimulation. A representative β-actin blot is shown showing equal loading in Fig.49.

Chemokine expression by MKN45 cells stimulated with cytomix

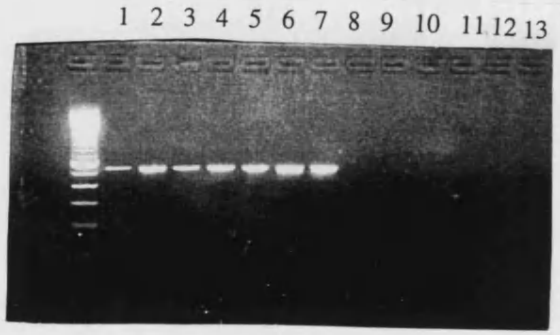


Fig.42 PCR analysis of IL-8/CXCL8 mRNA expression in MKN45 cells (562 bp)

1= Unstimulated MKN45 cells
2= MKN45 cells stimulated with cytomix for 1 hour
3= MKN45 cells stimulated with cytomix for 3 hours
4= MKN45 cells stimulated with cytomix for 6 hours
5 = MKN45 cells stimulated with cytomix for 12 hours
6= Positive control (HT29 cells stimulated with cytomix for 6 hours)
7 = Positive control (HT29 cells stimulated with cytomix for 12 hours)
8 = Water
9-13= samples of 1-5 without reverse transcriptase

This gel is representative of three independent experiments

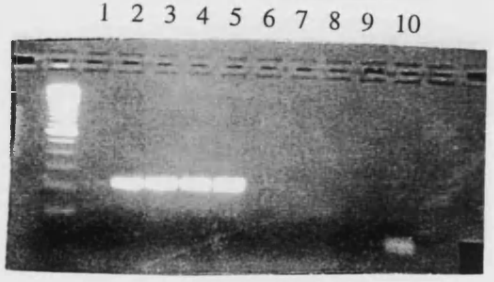


Fig. 43 PCR analysis of MIG/CXCL9 mRNA expression in MKN45 cells (214 bp)

1= Unstimulated MKN45 cells (very faint)
2= MKN45 cells stimulated with cytomix for 1 hour
3= MKN45 cells stimulated with cytomix for 3 hours
4= MKN45 cells stimulated with cytomix for 12 hours
5= Positive control (HT-29 stimulated with cytomix)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

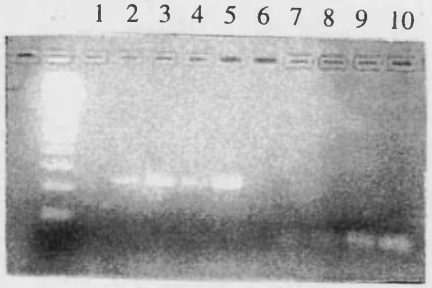


Fig.44 PCR analysis of IP-10/CXCL10 mRNA expression in MKN45 cells (229 bp)

1= Unstimulated MKN45 cells
2= MKN45 cells stimulated with cytomix for 1 hour
3= MKN45 cells stimulated with cytomix for 3 hours
4= MKN45 cells stimulated with cytomix for 12 hours
5= Positive control (HT29 stimulated with cytomix)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

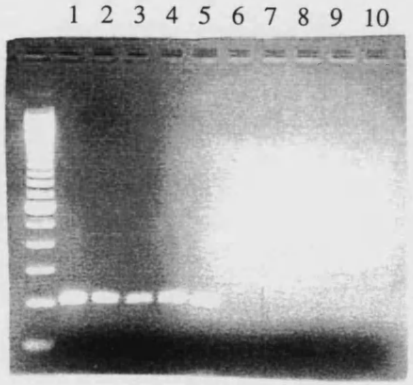


Fig.45 PCR analysis of LARC/CCL20 mRNA expression in MKN45 cells (210 bp)

1= Unstimulated MKN45 cells
2= MKN45 cells stimulated with cytomix for 1 hour
3= MKN45 cells stimulated with cytomix for 3 hours
4= MKN45 cells stimulated with cytomix for 12 hours
5= Positive control (White blood cells stimulated with PHA)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

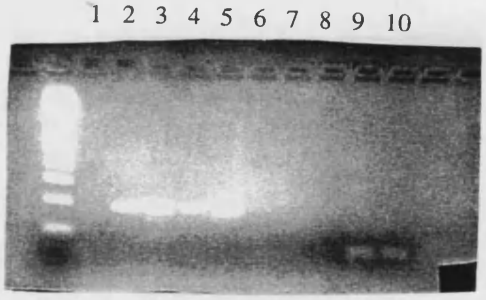


Fig.46 PCR analysis of MCP-1/CCL2 mRNA expression in MKN45 cells (177 bp)

1= Unstimulated MKN45 cells
2= MKN45 cells stimulated with cytomix for 1 hour
3= MKN45 cells stimulated with cytomix for 3 hours
4= MKN45 cells stimulated with cytomix for 12 hours
5= Positive control (HT29 stimulated with cytomix)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

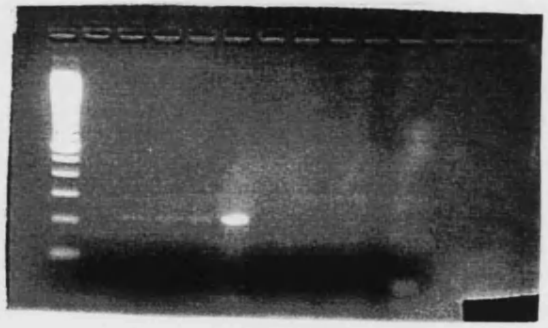


Fig. 47 PCR analysis of MCP-3/CCL7 mRNA expression in MKN45 cells (216 bp)

1= Unstimulated MKN45 cells
2= MKN45 cells stimulated with cytomix for 1 hour (very faint)
3= MKN45 cells stimulated with cytomix for 3 hours (very faint)
4= MKN45 cells stimulated with cytomix for 12 hours (very faint)
5= Positive control (U937 stimulated with PMA)
6-9= samples of 1-4 minus reverse transcriptase
10= water

This gel is representative of three independent experiments

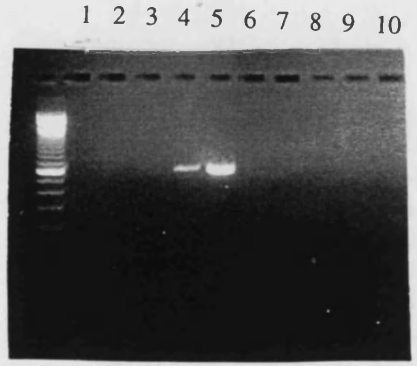


Fig. 48 PCR analysis of RANTES/CCL5 mRNA expression in the MKN45 cells (583 bp)

1 = Unstimulated MKN45 cells
2 = MKN45 cells stimulated with cytomix for 1 hour
3 = MKN45 cells stimulated with cytomix for 3 hours
4 = MKN45 cells stimulated with cytomix for 12 hours
5 = Positive control (U937 stimulated with PMA)
6-9 = samples of 1-4 minus reverse transcriptase
10 =Water

This gel is representative of three independent experiments

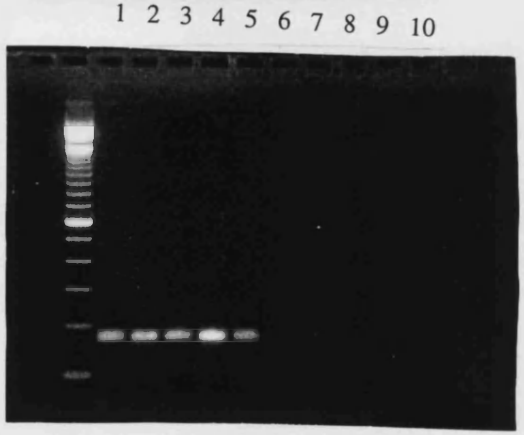


Fig. 49 PCR analysis of β-actin mRNA expression in the MKN45 cells (176 bp)

1 = Unstimulated MKN45 cells
2 = MKN45 cells stimulated with cytomix for 1 hour
3 = MKN45 cells stimulated with cytomix for 3 hours
4 = MKN45 cells stimulated with cytomix for 12 hours
5 = Positive control (HT29 cells stimulated with cytomix)
6-9 = samples of 1-4 minus reverse transcriptase
10 = Water

This gel is representative of three independent experiments

4.5 THE EXPRESSION OF NOVEL CHEMOKINES DETECTED IN GASTRIC BIOPSIES BY RT-PCR

A preliminary study was conducted on gastric biopsies collected from patients from the RNHRD Hospital to determine the expression of chemokines in patients with various disease conditions. The main limitation of this study was the small number of patients, however, preliminary data has been generated which will be useful for a larger study in the future involving a bigger sample size.

Gastric biopsies were obtained from patients who were divided into 3 categories, the normal patients, the patients with gastritis who were negative for the bacteria *H. pylori* and the patients with gastritis who were also positive for *H. pylori*.

The patients were diagnosed with their conditions using the rapid urease test ('CLO' test) and histological tests performed on slides of the gastric biopsies. The 'CLO' test detects urease produced by *H. pylori* which converts urea on the test medium to ammonia and results in a red colour shown by phenol red present on the test medium. Normal patients were characterised by endoscopy as those having normal antrum and body biopsies from the stomach without any inflammation or *H. pylori* infection. Patients with *H. pylori*-associated gastritis tended to have mild to moderate atrophy and inflammation in either the antrum and/or body sections of their stomach with a positive 'CLO' test indicating the presence of *H. pylori*. Patients with non-*H. pylori*-associated gastritis did not have any diagnosed *H. pylori* infections, yet their antral and gastric body biopsies still revealed active chronic superficial or quiescent gastritis, with or without atrophy.

Six patients were studied from the *H. pylori*-associated gastritis category, 4 patients were studied from the non- *H. pylori*-associated gastritis category and 4 patients from the normal category. Three or four biopsies were taken from the antrum and the body of the stomach. The biopsies contained many different types of cells such as epithelial cells, fibroblasts, smooth muscle cells, connective tissue cells, inflammatory cells and possibly some *H. pylori* bacteria. The gastric biopsies from each patient were examined separately for chemokine expression by 35 cycles of RT-

Table 6. The expression of chemokines in gastric biopsies by RT-PCR

Chemokine	Normal (n=4)	Normal (n=4)	Gastritis + *H. pylori* (n=6)	Gastritis + *H. pylori* (n=6)	Gastritis without *H. pylori* (n=4)	Gastritis without *H. pylori* (n=4)
CXC	**Antrum**	**Body**	**Antrum**	**Body**	**Antrum**	**Body**
IL-8/CXCL8	– – – –	– – – –	**++** – – – –	**++** – – – –	**+** – – –	– – – –
MIG/CXCL9	– – – –	– – – –	**+** – – – – –	**+** – – – – –	**++++**	**++** – –
IP-10/CXCL10	– – – –	– – – –	**+** – – – – –	**+** – – – – –	**++** – –	– – – –
SDF-1α/CXCL12	**+** – – –	**+** – – –	**+** – – – – –	**+** – – – – –	**++** – –	**+** – – –
BCA-1/CXCL13	– – – –	– – – –	**+** – – – – –	**+** – – – – –	**++** – –	**+** – – –
CC						
MCP-1/CCL2	**++** – –	**++++**	**+++** – – –	**+++** – – –	**+++** –	**+++** –
MIP-1α/CCL3	– – – –	– – – –	**++** – – – –	– – – – – –	**++** – –	**+** – – –
RANTES/CCL5	– – – –	– – – –	**+** – – – – –	– – – – – –	**++** – –	**+** – – –
MCP-3/CCL7	– – – –	– – – –	**+** – – – – –	**+** – – – – –	– – – –	– – – –
TARC/CCL17	– – – –	– – – –	– – – – – –	– – – – – –	– – – –	– – – –
LARC/CCL20	– – – –	– – – –	**+** – – – – –	**++** – – – –	**+++** –	**+++** –

NB. Each + sign symbolises a patient out of the total number of patients listed at the top of each column. The + sign which is in bold signifies a strong expression of the specific chemokine. The – sign signifies a negative expression of the specific chemokine.

PCR and the results tabulated (Table.6). Representative blots from the patients are shown in Figures 50-68. On all gels, a 100 base pair DNA ladder was used and the lanes were numbered from left to right.

The samples were run concurrently with a positive control and negative controls including water and a RT minus control for every sample. The cells used as positive controls were the same as those used in the RT-PCR experiments performed on the MKN45 and AGS cell lines (see section 4.4).

These experiments were performed to determine if *H. pylori* infection had any effect on the expression of chemokines in gastric mucosa and if the chemokine expression was dependent upon the presence of gastritis. In all experiments, the positive controls were positive and the negative controls were negative. A representative blot for β-actin, which is the housekeeping gene in all of the samples were included in each category. All cDNA derived from each sample was tested for the expression of the housekeeping gene, β-actin, indicating that cDNA was of acceptable quality and loaded equally in each experiment. These experiments were performed 2-3 times depending on the amount of mRNA extracted.

4.5.1 Normal patients

The polymerase chain reaction (PCR) experiments conducted on the gastric biopsies of 4 normal patients without gastritis revealed that chemokine expression was low. As shown in Table 6, the only chemokines detected were the monocyte chemoattractant MCP-1/CCL2(Fig.50) which was expressed in biopsies taken from the antrum and body in all 4 patients and SDF-1α/CXCL12 (Fig.51) which was detected in 1 out of the 4 patients. All other chemokines investigated were not detected (results not shown). Fig.52 represents a β-actin blot of the samples.

4.5.2 Patients with non-*H. pylori*-associated gastritis

Four patients were studied in this category. The chemokines expressed by many of the patients in this group were LARC/CCL20 (Fig.53), MCP-1/CCL2 (Fig.54) and

CHEMOKINES EXPRESSED BY A REPRESENTATIVE NORMAL PATIENT

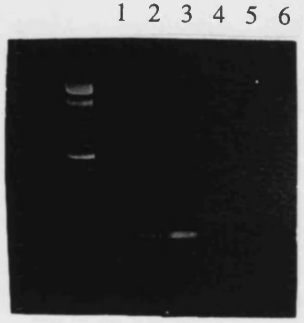


Fig. 50 PCR analysis of MCP-1/CCL2 mRNA expression (177 bp)

1 = gastric antrum
2 = gastric body
3 = positive control (HT-29 cells stimulated with cytomix)
4 = gastric antrum minus reverse transcriptase
5 = gastric body minus reverse transcriptase
6 = water
This gel is representative of three independent experiments

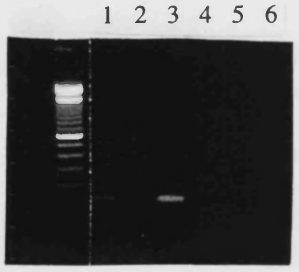


Fig. 51 PCR analysis of SDF-1α/CXCL12 mRNA expression (132 bp)

1 = gastric antrum
2 = gastric body
3 = positive control (fibroblasts)
4 = gastric antrum minus reverse transcriptase
5 = gastric body minus reverse transcriptase
6 = water
This gel is representative of three independent experiments. The DNA ladder was taken from another part of the same gel.

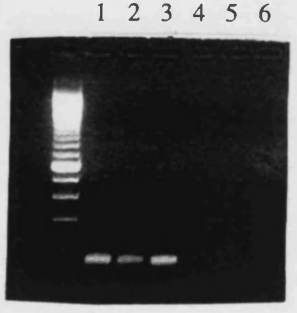


Fig. 52 PCR analysis of β-actin mRNA expression (176 bp)

1 = gastric antrum
2 = gastric body
3 = positive control (HT-29 stimulated with cytomix)
4 = gastric antrum minus reverse transcriptase
5 = gastric body minus reverse transcriptase
6 = water
This gel is representative of three independent experiments

MIG/CXCL9 (Fig.55). Many other chemokines were also expressed by some of the patients examined for example SDF-1α/CXCL12 (Fig.56), MIP-1α/CCL3 (Fig.57),

RANTES/CCL5 (Fig.58) and BCA-1/CXCL13 (Fig.59). A representative blot showing equal β-actin expression in all samples is shown in Fig.60. Three out of 4 patients in this category expressed the novel chemokine LARC/CCL20 in the antrum and gastric body, which was a majority of the patient numbers. Similar to the case of the patients with *H. pylori*-associated gastritis, LARC/CCL20 was expressed at higher levels in the gastric antrum compared to the gastric body, as was demonstrated by the bright PCR band on the gels. This emphasises the importance of LARC/CCL20 in the gastric mucosa. All of the patients expressed the chemokine MCP-1/CCL2 at apparently higher levels compared to normal patients. All of the patients expressed MIG/CXCL9 faintly in the antrum and 2 out of 4 patients expressed MIP-1α/CCL3, RANTES/CCL5, IP-10/CXCL10, SDF-1α/CXCL12 and BCA-1/CXCL13 mRNA in the antrum. IL-8/CXCL8 mRNA was only detected in the antrum of 1 patient. One interesting finding from these experiments was the observed expression of high levels of BCA-1/CXCL13 mRNA, which is a novel chemokine which attracts B lymphocytes, in half of the patients.

4.5.3 Patients with *H. pylori*- associated gastritis

Some of the 6 patients with *H. pylori*-associated gastritis expressed a multitude of chemokines. The chemokines expressed were BCA-1/CXCL13 (Fig.61), LARC/CCL20 (Fig.62), IP-10/CXCL10 (Fig.63), MIG/CXCL9 (Fig.64), MCP-1/CCL2 (Fig.65), IL-8/CXCL8 (Fig.66) and SDF-1α/CXCL12 (Fig.67). Only TARC/CCL17 was not expressed in samples taken from any of the patients. Most of the chemokines were expressed by the antrum. Interestingly, the chemokine expressed in 3 out of 6 patients in this category was LARC/CCL20, this is similar to the RT-PCR data generated from the AGS and MKN45 cell lines which also showed a significant expression of LARC/CCL20 mRNA in stimulated cells. MCP-1/CCL2 mRNA was also important as it was expressed in 3 out of the 6 patients in this category, however, from the normal patient category, it has been shown that MCP-1/CCL2 was expressed constitutively in the gastric body of all of the patients,

therefore MCP-1/CCL2 may not necessarily be induced by gastritis. As was seen in *H. pylori* negative gastritis samples, MCP-1/CCL2 and LARC/CCL20 mRNA were expressed in many patients with *H. pylori*. IL-8/CXCL8 mRNA was expressed in the antrum of 2 out of 6 patients with *H. pylori*-associated gastritis and this chemokine has been well-known to be expressed in inflammation and *H. pylori* infections as reported in previous studies. MIP-1α/CCL3 was expressed by 2 patients, while MCP-3/CCL7, RANTES/CCL5, MIG/CXCL9, IP-10/CXCL10 and SDF-1α/CXCL12 were expressed in 1 patient respectively. Fig.68 demonstrates the β-actin representative of the patients.

CHEMOKINES EXPRESSED BY A REPRESENTATIVE PATIENT WITH NON-*H. PYLORI* ASSOCIATED GASTRITIS

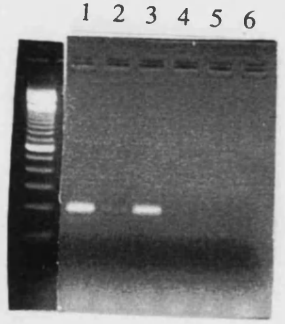


Fig.53 PCR analysis of LARC/CCL20 mRNA expression (210 bp)

1 = gastric antrum
2 = gastric body
3 = positive control (white blood cells stimulated with PHA)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water
This gel is representative of three independent experiments.
The DNA ladder was taken from another part of the same gel.

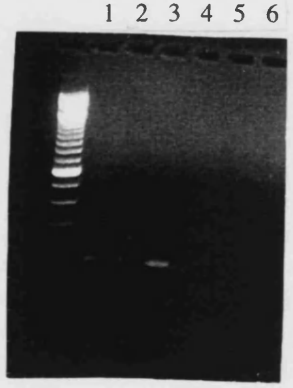


Fig.54 PCR analysis of MCP-1/CCL2 mRNA expression (177 bp)

1 = gastric antrum
2 = gastric body
3 = positive control (HT29 stimulated with cytomix)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water
This gel is representative of three independent experiments

CHEMOKINES EXPRESSED BY A REPRESENTATIVE PATIENT WITH NON-*H. PYLORI* ASSOCIATED GASTRITIS

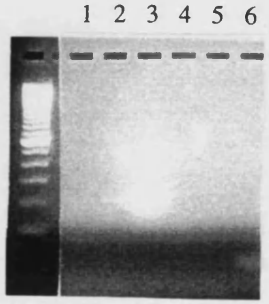


Fig.55 PCR analysis of MIG/CXCL9 mRNA expression (214 bp)

1 = gastric antrum (very faint)
2 = gastric body (very faint)
3 = positive control (HT29 stimulated with cytomix)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments. The DNA ladder was taken from another part of the same gel.

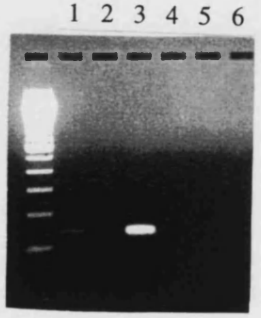


Fig.56 PCR analysis of SDF-1α/CXCL12 mRNA expression (132 bp)

1 = gastric antrum (very faint)
2 = gastric body
3 = positive control (fibroblasts)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments

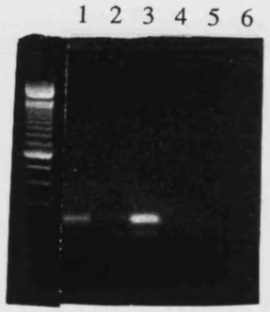


Fig.57 PCR analysis of MIP-1α/CCL3 mRNA expression (257 bp)

1 = gastric antrum
2 = gastric body
3 = positive control (white blood cells stimulated with PHA)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments. The DNA ladder was taken from another part of the same gel.

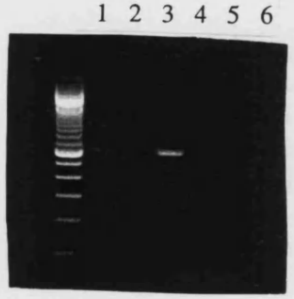


Fig.58 PCR analysis of RANTES/CCL5 mRNA expression (583 bp)

1 = gastric antrum (very faint)
2 = gastric body
3 = positive control (U937 stimulated with PMA)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments

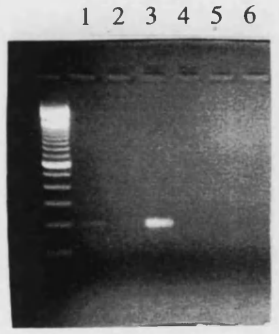


Fig.59 PCR analysis of BCA-1/CXCL13 mRNA expression (199 bp)

1 = gastric antrum (very faint)
2 = gastric body (very faint)
3 = positive control (white blood cells stimulated with PHA)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments

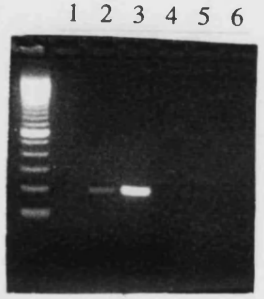
Fig. 60 PCR analysis of β-actin mRNA expression (176 bp)

1= gastric antrum
2= gastric body
3= positive control (HT29 cells stimulated with cytomix)
4= gastric antrum sample minus reverse transcriptase
5= gastric body sample minus reverse transcriptase
6= water

This gel is representative of three independent experiments

CHEMOKINES EXPRESSED BY A REPRESENTATIVE PATIENT WITH *H. PYLORI* ASSOCIATED GASTRITIS

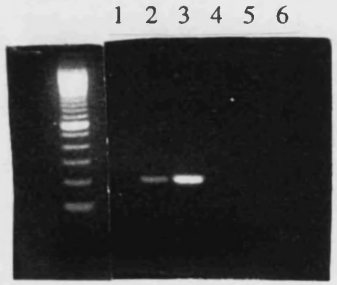
Fig. 61 PCR analysis of BCA-1/CXCL13 mRNA expression (199 bp)



1 = gastric antrum (very faint)
2 = gastric body
3 = positive control (white blood cells stimulated with PHA)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments

Fig.62 PCR analysis of LARC/CCL20 mRNA (210 bp)



1 = gastric antrum
2 = gastric body
3 = positive control (white blood cells stimulated with PHA)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments. The DNA ladder was taken from another part of the same gel.

Fig.63 PCR analysis of IP-10/CXCL10 mRNA expression (229 bp)

1 = gastric antrum (very faint)
2 = gastric body (very faint)
3 = positive control (HT-29 stimulated with cytomix)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments

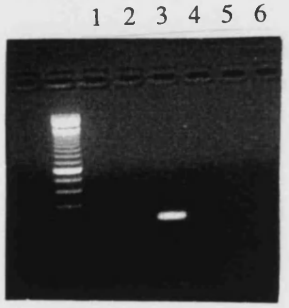


Fig. 64 PCR analysis of MIG/CXCL9 mRNA expression (214 bp)

1 = gastric antrum (very faint)
2 = gastric body (very faint)
3 = positive control (HT29 stimulated with cytomix)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments

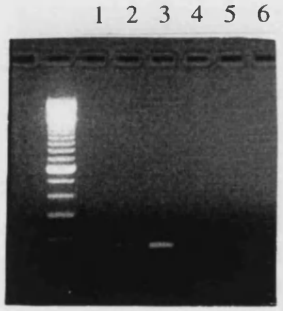


Fig.65 PCR analysis of MCP-1/CCL2 mRNA expression (177 bp)

1 = gastric antrum (very faint)
2 = gastric body (very faint)
3 = positive control (HT29 stimulated with cytomix)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments

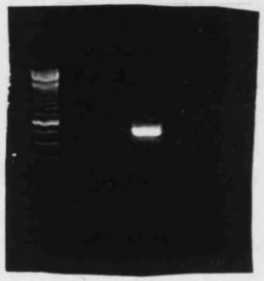


Fig.66 PCR analysis of IL-8/CXCL8 mRNA expression (562 bp)

1 = gastric antrum (very faint)
2 = gastric body (very faint)
3 = positive control (HT29 stimulated with cytomix)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments

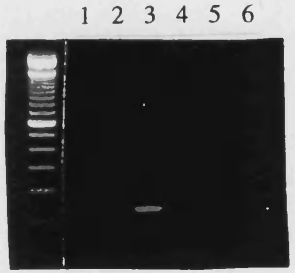


Fig.67 PCR analysis of SDF-1α/CXCL12 mRNA expression (132 bp)

1 = gastric antrum (very faint)
2 = gastric body (very faint)
3 = positive control (fibroblasts)
4 = gastric antrum sample minus reverse transcriptase
5 = gastric body sample minus reverse transcriptase
6 = water

This gel is representative of three independent experiments.
The DNA ladder was taken from another part of the same gel.

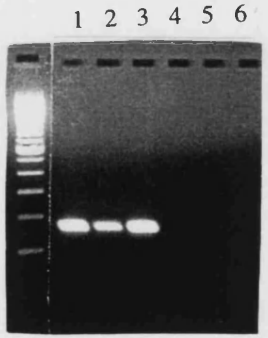


Fig. 68 PCR analysis of β-actin mRNA expression (176 bp)

1 = gastric antrum
2 = gastric body
3 = positive control (HT29 stimulated with cytomix)
4 = gastric antrum minus reverse transcriptase
5 = gastric body minus reverse transcriptase
6 = water

This gel is representative of three independent experiments.
The DNA ladder was taken from another part of the same gel.

In conclusion, these experiments demonstrated the detection of a larger number of different chemokines in patients with gastritis compared to the 'normal' patients. Since LARC/CCL20 was not detected in gastric samples from 'normal' patients, but was expressed in many of the patients with gastritis, this suggests that it may be up-regulated in gastric epithelium in response to inflammation.

4.6 THE EXPRESSION OF CHEMOKINE RECEPTORS ON AGS AND MKN45 GASTRIC EPITHELIAL CELL LINES

The chemokine receptors investigated included a range of 'CXC' and 'CC' chemokine receptors including those known to be the receptors for the chemokines found to be expressed by the AGS and MKN45 cell lines. The receptors studied for the 'CXC' chemokines were CXCR1-5 and the receptors studied for the 'CC' chemokines were CCR1,2,3,4,5 and 7. The chemokine receptor expression was investigated in unstimulated cells in the absence of foetal bovine serum to study the constitutive expression of chemokine receptors. To investigate whether pro-inflammatory cytokines could modify chemokine receptor expression, analysis of CCR7, CXCR1 and CXCR5 expression in AGS cells and CCR2 and CXCR5 expression in MKN45 cells was additionally performed using cells which had been stimulated for 1, 3 and 12 hours with cytomix. A positive control was included to validate primer pairs and was made up of unstimulated white blood cells or U937 cells stimulated with PMA. Each experiment was repeated at least two times and representative blots have been shown. On all gels, a 100 base pair DNA ladder was used and the lanes were numbered from left to right. The results from these experiments are tabulated in Table 7.

Table 7: Chemokine receptors expressed expressed by the AGS and MKN45 gastric epithelial cell lines

Chemokine receptor	AGS (un-stimulated)	AGS (+ 'cytomix')	MKN45 (un-stimulated)	MKN45 (+ 'cytomix')
CXCR1	+	+++ (#)	-	N/A
CXCR2	-	N/A	-	N/A
CXCR3	-	N/A	-	N/A
CXCR4	-	N/A	++ (*)	N/A
CXCR5	-	-	+ (#)	-
CCR1	+ (#)	N/A	-	N/A
CCR2	+ (#)	N/A	-	+ (#)
CCR3	-	N/A	-	N/A
CCR4	-	N/A	-	N/A
CCR5	-	N/A	-	N/A
CCR6	-	N/A	-	N/A
CCR7	-	N/A	-	N/A

+++ = very strong signal; ++ = strong signal; + = weak signal; - = not detected
N/A = not available; * = potentially important; # = preliminary data

The unstimulated AGS cell line did not express any chemokine receptors except low levels of CCR1 (Fig.69), CCR2 (Fig.70) and CXCR1 (Fig.75). CXCR2 (Fig.76), CXCR3 (Fig.77), CXCR4 (Fig.78) and CXCR5 (Fig.79) were not detected in AGS cells. CXCR1 was detected in unstimulated cells and the expression increased in response to 1, 3 and 12 hour stimulation with pro-inflammatory cytokines. CXCR5 expression was not induced by cytokines.

PCR analysis of unstimulated MKN45 cells demonstrated that these cells expressed low levels of CCR2 (Fig.81) and CXCR5 (Fig.87) as shown by very faint bands on the gels with relatively high expression of CXCR4 as shown by a bright band on the gel (Fig.86) which was detected reproducibly in 3 separate experiments. CCR1 (Fig.80), CCR3 (Fig.82) and CXCR1 (Fig.83), CXCR2 (Fig.84) and CXCR3 (Fig.85) mRNA were not detected in the MKN45 cells. The primers were known to work as positive control bands were seen on the gel, although with different grades of brightness for different primers used.

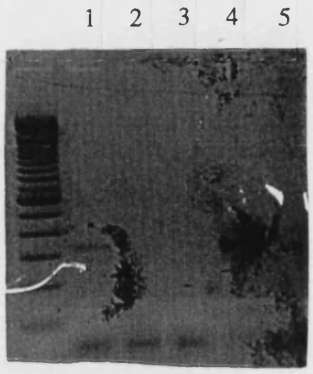
In conclusion, in this study, most of the chemokine receptors were not shown to be expressed in unstimulated gastric epithelial cell lines. Low expression of CCR2 in both the AGS and MKN45 cell lines may be important as CCR2 is the receptor for MCP-1/CCL2 and MCP-1/CCL2 mRNA has been shown to be expressed in gastric biopsies (section 4.5). CXCR5 may also play an important role since its ligand, BCA-1/CXCL13 was shown by immunohistochemistry to be expressed in gastric epithelial cells (section 4.7). The detection of high levels of CXCR4 in MKN45 cells is similar to the results obtained in HT-29 colonic epithelial cells (Jordan et al., 1999), where CXCR4 was the only chemokine receptor detected.

4.7 IMUNOHISTOCHEMICAL STAINING FOR CHEMOKINES AND CHEMOKINE RECEPTORS IN GASTRIC BIOPSIES

Paraffin-embedded sections of human gastric biopsies taken from the gastric antrum and body of patients with different pathological conditions were stained with antibodies for the CXC chemokine receptor, CXCR4 and its corresponding ligand, SDF-1α/CXCL12. The slides were also stained for the novel chemokine, LARC/CCL20 mRNA, since the mRNA for this chemokine had been detected earlier

PCR analysis of the chemokine receptor mRNA expression by the AGS cells

Fig.69

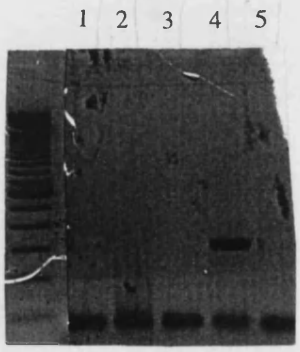


CCR1 (327bp)

1 = AGS (very faint)
2 = AGS RT minus
3 = water
4 = unstimulated white blood cells
5 = white blood cells stimulated with PHA and LPS

This gel is representative of three independent experiments

Fig.70

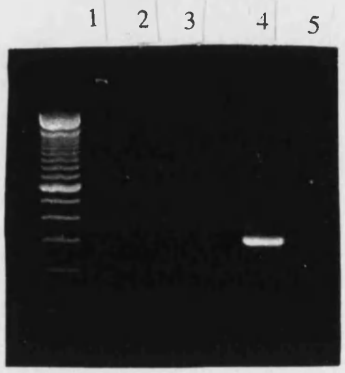


CCR2 (255bp)

1 = AGS (very faint)
2 = AGS RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of three independent experiments. The DNA ladder was taken from another part of the same gel.

Fig.71



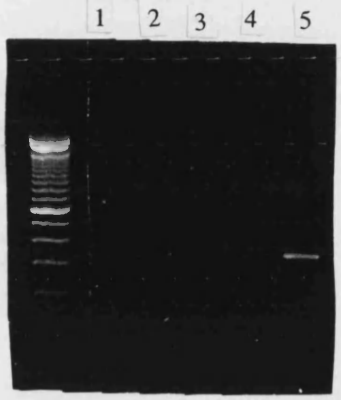
CCR3 (315bp)

1 = AGS
2 = AGS RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments

PCR analysis of the chemokine receptors mRNA in the AGS cells

Fig.72

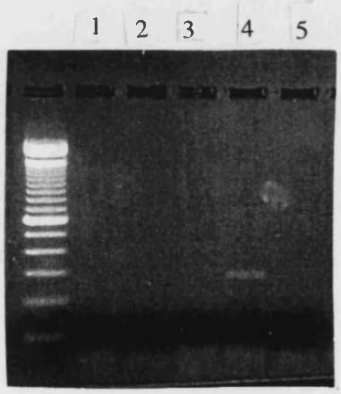


CCR4 (349 bp)

1 = AGS
2 = AGS RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments.
The DNA ladder was taken from another part of the same ge

Fig.73



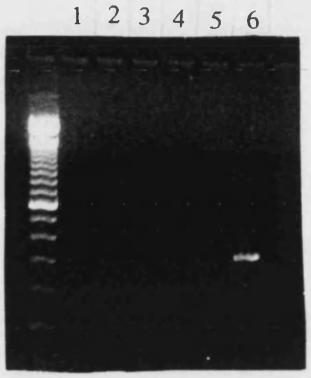
CCR5 (280 bp)

1 = AGS
2 = AGS RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments

PCR analysis for the chemokine receptors mRNA expressed by the AGS cells

Fig.74

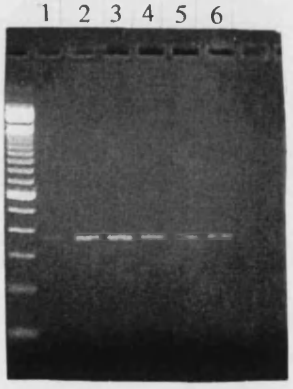


CCR7 (362 bp)

1 = Unstimulated AGS
2 = AGS stimulated with cytomix for 1 hour
3 = AGS stimulated with cytomix for 3 hours
4 = AGS stimulated with cytomix for 12 hours
5 = U937 stimulated with PMA
6 = White blood cells stimulated with PHA

This gel is representative of two independent experiments

Fig.75

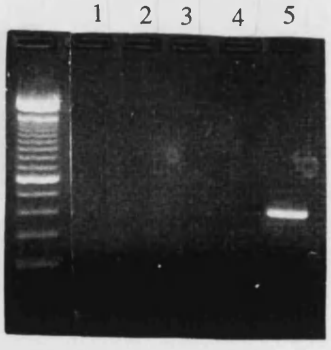


CXCR1 (363 bp)

1 = Unstimulated AGS
2 = AGS stimulated with cytomix for 1 hour
3 = AGS stimulated with cytomix for 3 hours
4 = AGS stimulated with cytomix for 12 hours
5 = U937 stimulated with PMA
6 = White blood cells stimulated with cytomix

This gel is representative of three independent experiments. The DNA ladder was taken from another part of the same gel.

Fig.76



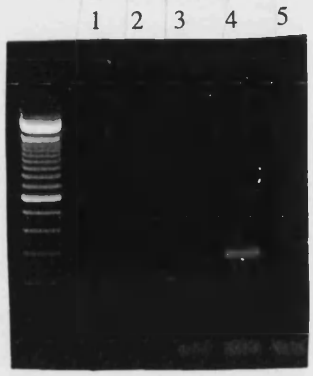
CXCR2 (385 bp)

1 = AGS
2 = AGS RT minus
3= water
4 = Unstimulated white blood cells
5= White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments

PCR analysis for the chemokine receptors mRNA expressed by the AGS cell line

Fig. 77

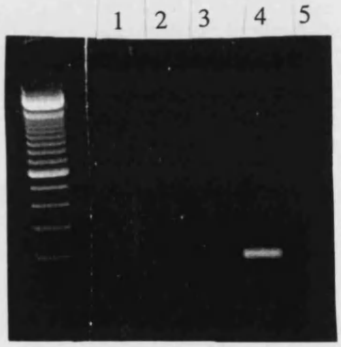


CXCR3 (293 bp)

1 = Unstimulated AGS
2 = AGS RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments.

Fig.78

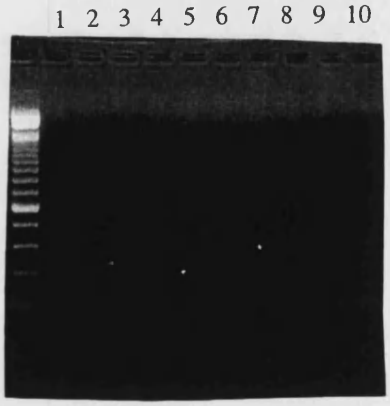


CXCR4 (206 bp)

1 = Unstimulated AGS
2 = AGS RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments.
The DNA ladder was taken from another part of the same gel.

Fig. 79

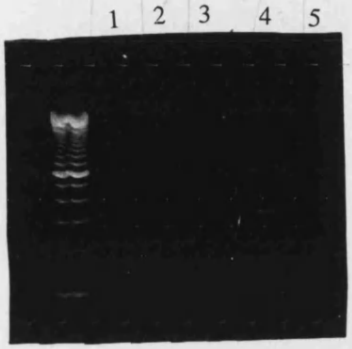


CXCR5 (465 bp)

1 = Unstimulated AGS
2 = AGS stimulated with cytomix for 1 hour
3 = AGS stimulated with cytomix for 3 hours
4 = AGS stimulated with cytomix for 12 hours
5 = U937 stimulated with PMA (very faint)
6 = Unstimulated AGS RT minus
7 = AGS stimulated with cytomix for 1 hour RT minus
8 = AGS stimulated with cytomix for 3 hours RT minus
9 = AGS stimulated with cytomix for 12 hours RT minus
10 = Water
This gel is representative of two independent experiments.

PCR analysis of chemokine receptors mRNA expressed by the MKN45 cell line

Fig.80

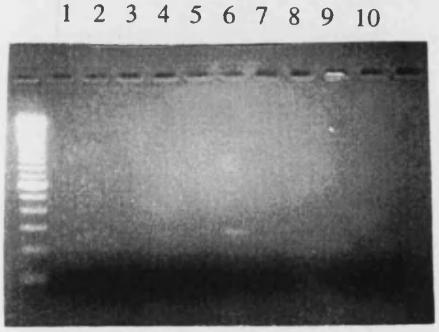


CCR1 (327 bp)

1 = Unstimulated MKN45
2 = Unstimulated MKN45 RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments

Fig.81

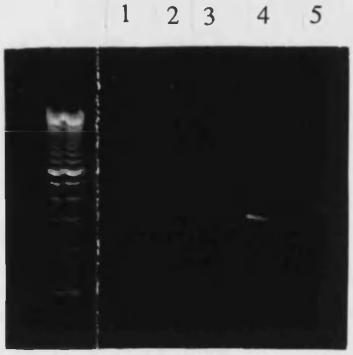


CCR2 (255 bp)

1 = Unstimulated MKN45 cells
2 = MKN45 stimulated with cytomix for 1 hour (very faint)
3 = MKN45 stimulated with cytomix for 3 hours
4 = MKN45 stimulated with cytomix for 12 hours (very faint)
5 = White blood cells stimulated with PHA
6 = U937 cells stimulated with PMA (very faint)
7 = Unstimulated MKN45 cells RT minus
8 = MKN45 stimulated with cytomix for 1 hour RT minus
9 = MKN45 stimulated with cytomix for 3 hours RT minus
10= MKN45 stimulated with cytomix for 12 hours RT minus
11 = water

This gel is representative of three independent experiments

Fig.82



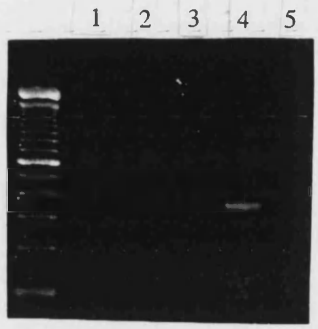
CCR3 (315 bp)

1 = Unstimulated MKN45
2 = Unstimulated MKN45 RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments. The DNA ladder was taken from another part of the same gel.

PCR analysis of the chemokine receptors mRNA expression by the MKN45 cell line

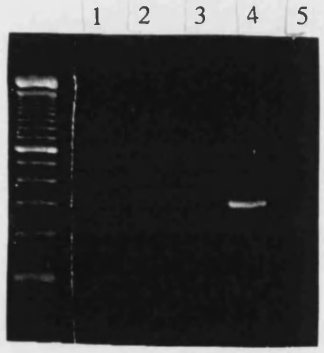
Fig.83 **CXCR1 (363 bp)**



1 = Unstimulated MKN45 cells
2 = Unstimulated MKN45 cells RT minus
3 = water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments

Fig.84 **CXCR2 (385 bp)**



1 = Unstimulated MKN45 cells
2 = Unstimulated MKN45 cells RT minus
3 = water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments.
The DNA ladder was taken from another part of the same gel.

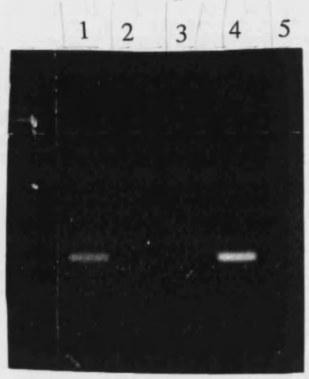
Fig.85 **CXCR3 (293 bp)**

1 = Unstimulated MKN45 cells
2 = Unstimulated MKN45 cells RT minus
3 = water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of two independent experiments

PCR analysis of the chemokine receptors mRNA expressed by the MKN45 cells

Fig.86

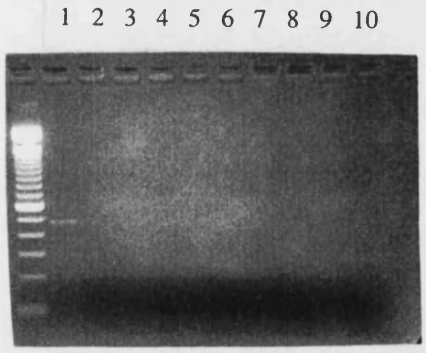


CXCR4 (206 bp)

1 =Unstimulated MKN45 cells
2 = Unstimulated MKN45 cells RT minus
3 = Water
4 = Unstimulated white blood cells
5 = White blood cells stimulated with PHA and LPS

This gel is representative of three independent experiments.
The DNA ladder was taken from another part of the same gel.

Fig.87



CXCR5 (465 bp)

1 = Unstimulated MKN45 cells (very faint)
2 = MKN45 stimulated with cytomix for 1 hour
3 = MKN45 stimulated with cytomix for 3 hours
4 = MKN45 stimulated with cytomix for 12 hours
5 = White blood cells stimulated with PHA (very faint)
6 = U937 cells stimulated with PMA
7 = Unstimulated MKN45 cells RT minus
8 = MKN45 stimulated with cytomix for 1 hour RT minus
9 = MKN45 stimulated with cytomix for 3 hours RT minus
10 = MKN45 stimulated with cytomix for 12 hours RT minus
11 = Water

This gel is representative of three independent experiments

in this study in AGS and MKN45 gastric epithelial cell lines and in inflamed human gastric biopsies in patients with *H. pylori*-associated gastritis and non-*H. pylori*-associated gastritis.

Immunohistochemistry staining was also performed on biopsy sections for BCA-1/CXCL13. BCA-1/CXCL13 mRNA had been shown previously by RT-PCR to be expressed by patients with *H. pylori*-associated gastritis and non-*H. pylori*-associated gastritis.

Staining of the slides from each patient were usually carried out in duplicate. One slide was stained with the specific antibody and the other slide from the same patient was stained in exactly the same way with no antibody used. The second slide provided a negative control. This control indicated that the primary antibody used did not stain non-specifically.

The SDF-1α/CXCL12 and LARC/CCL20 antibodies did not give any specific staining on any of the slides investigated. This was the first known time that LARC/CCL20 staining was attempted on human gastric tissues. The absence of positive LARC/CCL20 staining suggests that LARC/CCL20 protein is not expressed in the tissues examined. Our previous results obtained from gastric biopsies indicated that LARC/CCL20 mRNA was expressed, therefore it is possible that this is not translated into detectable quantities of protein in gastric tissue. An alternative explanation could be that the antibody used was not suitable for immunohistochemical detection of LARC/CCL20. Unfortunately, no other anti-human LARC/CCL20 antibodies tested in immunohistochemistry were commercially available at the time these experiments were carried out. Different concentrations of the SDF-1α/CXCL12 and LARC/CCL20 antibodies were used in an attempt to improve results. A different method of antigen retrieval other than the pressure-cooking method in which the slides were treated with Pronase enzyme was also tried. However, despite using this other technique, no staining was observed for LARC/CCL20. The lack of specific staining for SDF-1α/CXCL12 in our studies, could possibly be explained by the absence of a suitable anti-human SDF-1α/CXCL12 antibody in our laboratory.

In the immunohistochemical analysis of CXCR4, slides from 9 patients with *H. pylori*-associated gastritis, 7 patients with non-*H. pylori*-associated gastritis, 2 patients with adenocarcinoma and 1 normal patient were stained. Immunohistochemical staining was performed for CXCR4 since this receptor was expressed in the gastric epithelial cell line, MKN45 (see section 4.4). The aim of immunohistochemical analysis was to determine if 'real' gastric epithelial cells in human gastric mucosa could be identified to express CXCR4 protein.

Histological slides were successfully stained with the antibody for the chemokine receptor CXCR4. Slides representative of each pathological category such as normal and healthy patients, patients with adenocarcinoma, patients with *H. pylori*-associated gastritis and patients with non-*H. pylori*-associated gastritis were stained. The positively stained cells were stained brown in colour, whereas unstained cells appeared blue since these cells did not take up the DAB chromogen colour. The inflammatory cells made up of probably lymphocytes, macrophages and plasma cells found in the lamina propria of the gastric mucosa also stained positively for CXCR4.

An interesting observation made was that in the antrum, the mucosa were made up of gastric glands which were loosely packed and these glands occupied half of the gastric mucosal thickness. The antral mucosa also contains glands with a bubbly or foamy appearance. These glands mostly secreted mucin only and it was not common to find chief cells in these glands, although parietal cells could be present either singly or in small groups.

In contrast, the mucosa in the gastric body are made up of gastric glands which are tightly packed and straight. The cells in the gastric body consist of chief cells at the base, parietal cells further up the gland and mucous neck cells at the upper part of the glands. Therefore the gastric gland in the gastric body is made up of specific cell types.

In the gastric body, although CXCR4 was found to be expressed strongly on the surface epithelial cells, diffuse positive staining for CXCR4 was also found throughout the gastric glands. This is shown in Fig.88(a). This biopsy was obtained from a patient with *H. pylori* positive active chronic superficial gastritis.

Interestingly, most of the positive staining for CXCR4 in the antrum was observed to be in the surface epithelial cells. The staining for CXCR4 decreased further down the gastric glands. This is best demonstrated by Fig.89(a) which was taken from the antrum of a patient with *H. pylori* negative reactive gastritis.

CXCR4 was observed in gastric epithelial cells found in the gastric glands in patients with *H. pylori*-associated gastritis (Fig.88a), non-*H. pylori*-associated gastritis (Fig.89a), adenocarcinoma (Fig.91) and normal patients (Fig.90). The most dense staining for CXCR4 was observed in histological slides taken from patients with *H. pylori*-associated gastritis (Fig.88a). No *H. pylori* bacteria were visibly present in these slides. In all of these positively-stained slides, there was a little background staining in the lamina propria area and this was probably due to the scattered presence of CXCR4 in the inflammatory cells in the lamina propria, although the majority of the cells which were positively-stained for CXCR4 were the gastric epithelial cells. The positive staining for CXCR4 in the patients with adenocarcinoma was parallel with our detection of CXCR4 mRNA in the MKN45 cell line (section 4.6), since this is a gastric epithelial cell line derived from an adenocarcinoma. In the patients with adenocarcinoma, even the abnormal gastric epithelial cells within the area of the tumour stained positively for CXCR4. These results implied that CXCR4 was up-regulated in tissues which were undergoing either inflammatory processes such as in gastritis and also tissues which were undergoing neoplastic changes into tumour tissues.

Immunohistological staining of patients slides for BCA-1/CXCL13 were also carried out. In this experiment, the number of patients studied were 1 patient with 'MALT'oma, 2 patients with *H. pylori*-associated gastritis, 2 patients with non-*H. pylori*-associated gastritis and 1 normal patient. In these experiments, positively stained slides appeared brown, which was the colour of the DAB chromogen which stained the gastric tissues, while all the negatively-stained slides appeared blue. Similar to the immunohistochemical experiments for CXCR4, the inflammatory cells in the lamina propria also stained positively for BCA-1/CXCL13.

In the antral mucosa, positive staining for BCA-1/CXCL13 was observed in the upper part of the gastric glands with a gradual decrease further down the gastric

gland. Similar as for the positive staining for CXCR4, the gastric body demonstrated a diffuse positive staining for BCA-1/CXCL13 throughout the gastric glands from the surface epithelial cells to the cells at the base of the gastric glands.

Sections from the normal patient (without gastritis) (Fig.92a) showed low levels of positive staining for BCA-1/CXCL13. This suggested very low constitutive BCA-1/CXCL13 was expressed in normal patients. The BCA-1/CXCL13 staining appeared in the gastric epithelial cells. The corresponding negative control slide (Fig.92b) for the normal patient was totally blue, showing that the antibody used was not staining non-specifically.

Faint positive staining for BCA-1/CXCL13 was also detected in the sections from the patients with non-*H. pylori*-associated gastritis (Fig.94a). The negative control showed no staining (Fig.94b). These sections were taken from the gastric antrum as demonstrated by the less dense arrangement of the gastric glands and the bubbly and foamy appearance of the gastric glands. The positive staining also appeared mainly in the gastric epithelial cells in the upper region of the gastric glands. Some staining was also found in the lamina propria, although this was to a lesser degree compared to the epithelial cells in the gastric glands.

Very dense staining was present in the gastric epithelial cells of the gastric glands in the patients with *H. pylori*-associated gastritis (Fig. 93a). The negative control for this section is stained blue and is shown in Fig.93(b). These sections were taken from the gastric body as shown by the appearance of the gastric glands which are very tightly packed. The epithelial cells in the gastric glands were stained very strongly for BCA-1/CXCL13. Some positive staining was observed in the lamina propria although very strong and diffuse staining for BCA-1/CXCL13 was observed in the lymphoid follicle in the gastric mucosa as BCA-1/CXCL13 attracts B lymphocytes and there are many B lymphocytes in the lymphoid follicle. The presence of the lymphoid follicle is indicative of *H. pylori* infection.

Positive staining for BCA-1/CXCL13 was also observed in the gastric epithelial cells in Fig.94(a). This slide was taken from a patient with *H. pylori*-negative chronic reactive gastritis. Interestingly, the staining was not as dense as that observed in the

gastric epithelial cells in Fig.93(a) which was taken from a patient infected with the *H. pylori* bacteria. However, the staining in Fig.94(a) was darker than the positive staining seen in a normal patient (Fig.92(a). This suggests that BCA-1/CXCL13 expression was elevated in gastritis and inflammatory states in the stomach compared to the levels normally expressed constitutively. *H. pylori* infection may possibly also play a role in increasing the expression of BCA-1/CXCL13. Inflammatory cells in the lamina propria also demonstrated positive staining for BCA-1/CXCL13 in slides 92(a), 93(a), 94(a) and 95(a), although the most dense staining was observed in the slide taken from the patient with *H. pylori*-associated gastritis.

Similarly, very dense staining for BCA-1/CXCL13 was observed in the patient with *H. pylori*-induced 'MALT'oma lymphoma (mucosal-associated lymphoid tissue lymphoma) (Fig.95a). *H. pylori* infection is marked by the presence of the lymphoid follicle. Very dense staining for BCA-1/CXCL13 was observed in the lymphoid follicle suggesting there was an accummulation of BCA-1/CXCL13 within the area of the lymphoid follicle.where B lymphocytes were present (Fig.95a). There was also some lighter positive staining for BCA-1/CXCL13 in the gastric epithelial cells as well as in the inflammatory cells in the lamina propria. Fig. 95(b) is the negative control slide for this patient with 'MALT'oma lymphoma.

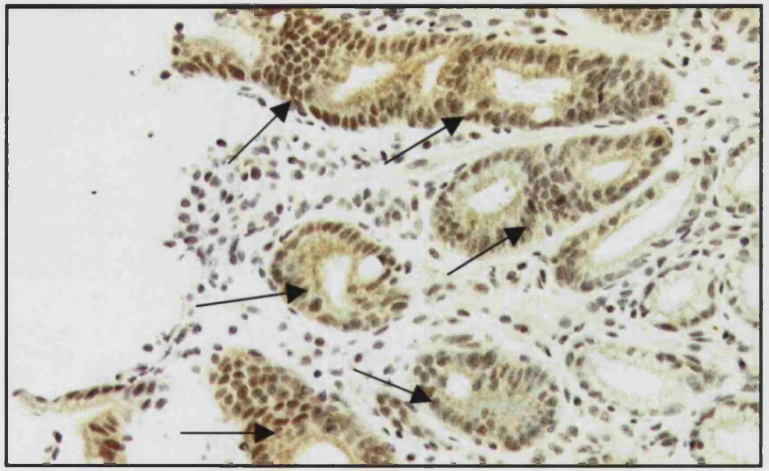
In conclusion, these immunohistochemistry experiments successfully demonstrated positive staining for the CXCR4 chemokine receptors in normal and inflamed gastric tissues, where it was strongly expressed by the gastric epithelial cells in patients who had *H. pylori*-associated gastritis. CXCR4 was also expressed to a lesser degree in patients with non-*H. pylori*-associated gastritis. CXCR4 was also expressed in abnormal gastric tissues as in adenocarcinoma. This supports the detection for constitutive and induced CXCR4 mRNA in gastric epithelial cells.

BCA-1/CXCL13 positive staining was also observed in small numbers of gastric epithelial cells in normal patients suggesting some constitutive expression of this chemokine in these cells. Both the staining for CXCR4 and BCA-1/CXCL13 were more diffuse for gastric tissues which were undergoing inflammatory processes such as gastritis and carcinogenesis including 'MALT'oma. Interestingly, *H. pylori* also seemed to play an important role in causing stronger expression of CXCR4 and

BCA-1/CXCL13 in the gastric tissues of patients with *H. pylori*-associated gastritis. These can be seen by very dense staining for CXCR4 and BCA-1/CXCL13 in the sections where the *H. pylori* infection is present.

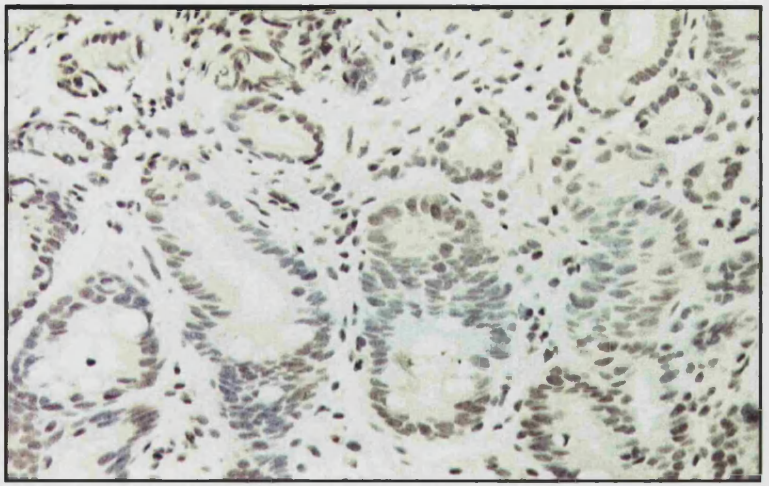
Sections of biopsy samples taken from human gastric mucosa

Fig.88(a)



Taken from a patient (15741) with *H. pylori* positive active chronic superficial gastritis. Immunostaining with anti-human CXCR4. Magnification X200. Positive staining was observed in the gastric epithelial cells in the gastric glands as shown by the arrows.

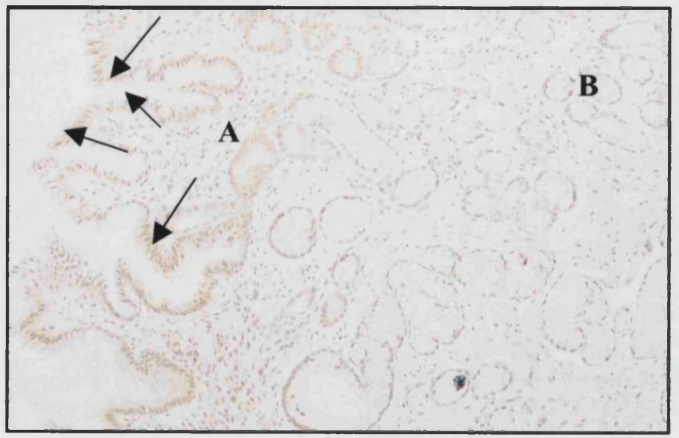
Fig.88(b)



Taken from a patient (15741) with *H. pylori* positive active chronic superficial gastritis. Negative control for Fig.88(a). No staining was observed when the primary anti-human CXCR4 was omitted. Magnification X200.

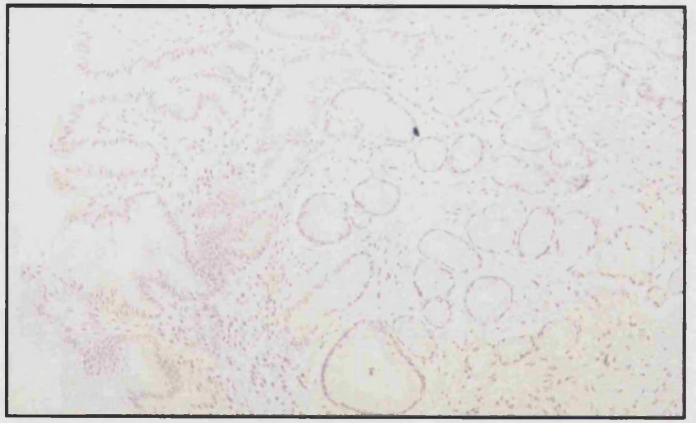
Sections of biopsy samples taken from human gastric mucosa

Fig.89(a)



Taken from the gastric antrum of a patient (8170) with *H. pylori* negative reactive gastritis. Immunostaining with anti-human CXCR4. Magnification X100. Very dense staining for CXCR4 was observed in the surface gastric epithelial cells (top left hand corner) (A) as shown by the arrows. The staining for CXCR4 was fainter further down in the gastric glands (B).

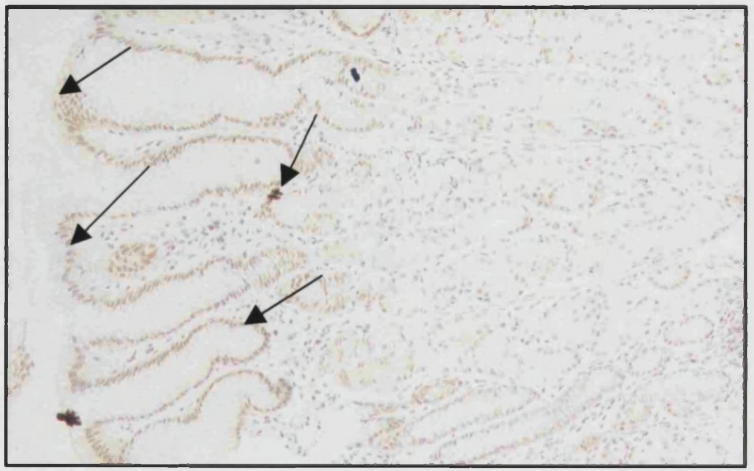
Fig.89(b)



Taken from the gastric antrum of a patient (8170) with *H. pylori* negative reactive gastritis. Negative control for Fig.(89a). No staining was observed when the primary anti-human CXCR4 was omitted. Magnification X100.

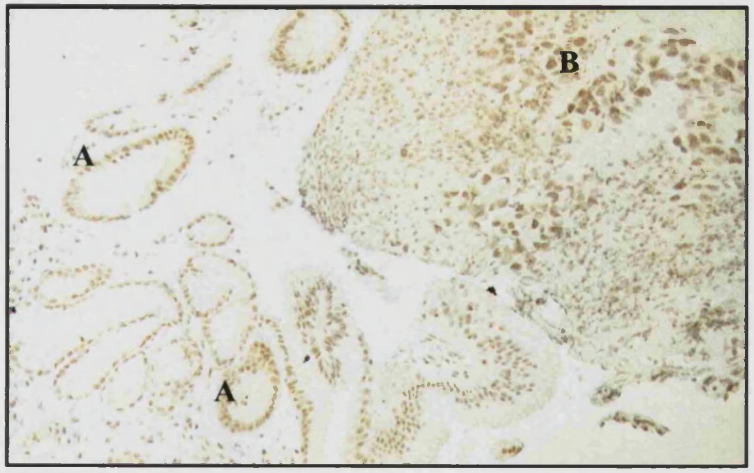
Sections of biopsy samples taken from human gastric mucosa

Fig.90



Taken from the gastric body of a normal patient (2719). Immunostaining with anti-human CXCR4 antibody. Magnification X100. Very dense staining for CXCR4 was observed in the surface gastric epithelial cells (on the left side of the photograph-see arrows) and very light positive staining for CXCR4 was observed further down the gastric glands (right side of the photograph).

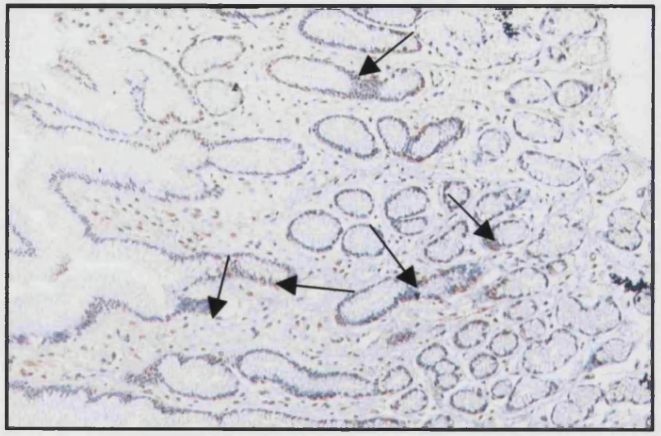
Fig.91



Taken from a patient (954) with adenocarcinoma. Immunostaining with anti-human CXCR4. Magnification X100. Positive staining for CXCR4 was observed in the gastric epithelial cells in the gastric glands (left side of the photograph marked A) and in the area of abnormal-looking gastric epithelia to the right of the photograph marked B).

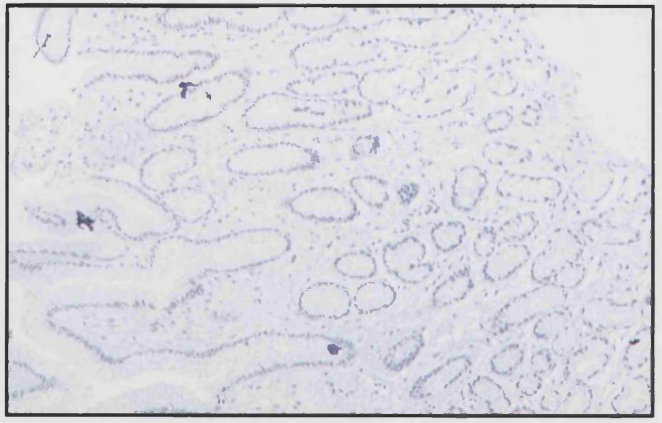
Sections of biopsy samples taken from human gastric mucosa

Fig.92(a)



Taken from the gastric antrum in a normal patient (9813). Immuno-staining with anti-human BCA-1/CXCL13 antibody. Magnification X100. Some light brown positive staining for BCA-1/CXCL13 was observed in the gastric glands in the middle of the photograph (see arrows), as well as some lighter staining present on the surface gastric epithelial cells (to the left side of the photograph). Some inflammatory cells in the lamina propria also stained positively for BCA-1/CXCL13, although this staining was rather light.

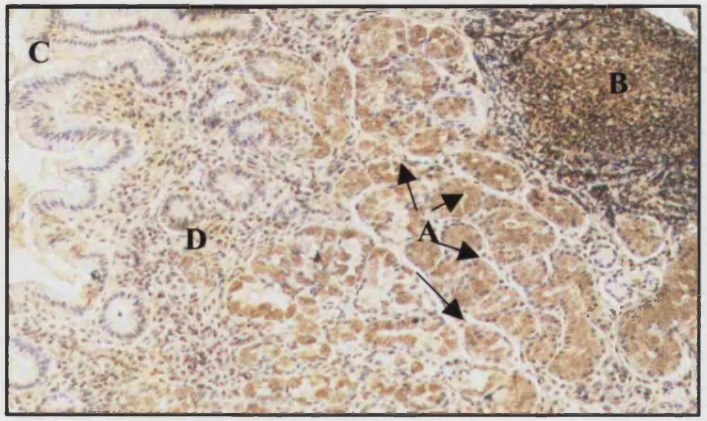
Fig.92(b)



Taken from the gastric antrum in a normal patient (9813). Negative control for Fig. 92(a). No staining was observed when the primary anti-human BCA-1/CXCL13 antibody was omitted. Magnification X 100.

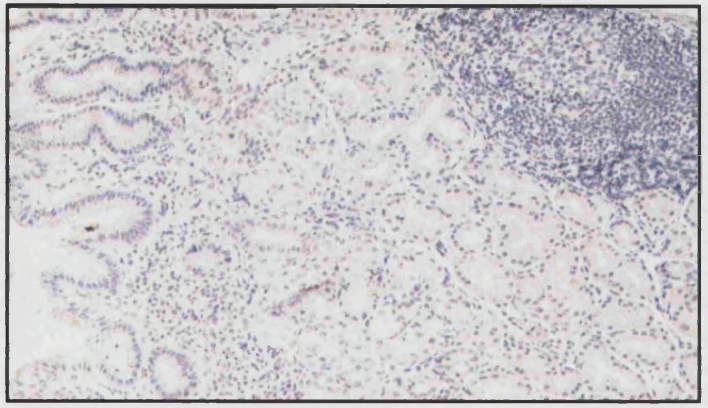
Sections of biopsy samples taken from human gastric mucosa

Fig.93(a)



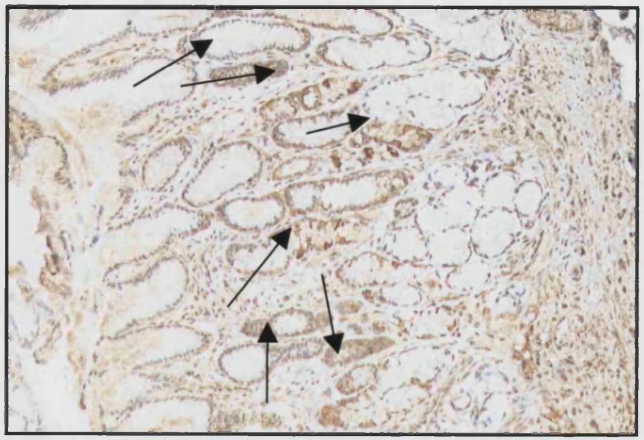
Taken from the gastric body of a patient (10630) with *H. pylori*-positive active superficial gastritis. Immunostaining with anti-human BCA-1/CXCL13 antibody. Magnification X100. Very heavy staining for BCA-1/CXCL13 throughout the gastric glands (A) (see arrows), lymphoid follicle (B) in the top right corner of the photograph and in the surface gastric epithelial cells (C). Some positive staining is also present due to inflammatory cells in the lamina propria (D).

Fig.93(b)



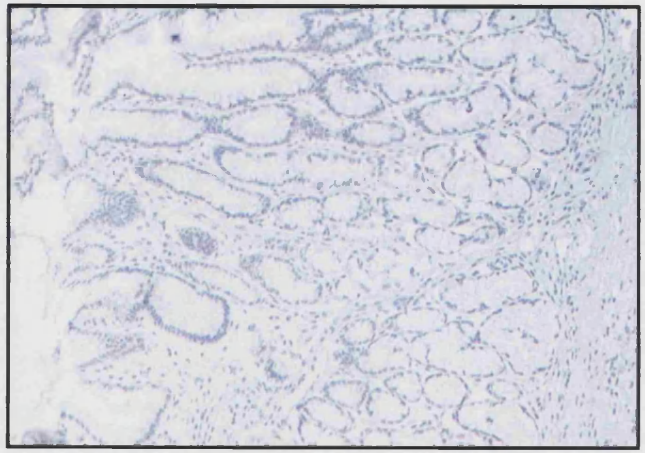
Taken from the gastric body of a patient (10630) with *H. pylori*-positive active superficial gastritis. Negative control for Fig. (93a). No staining was observed when the primary anti-human BCA-1/CXCL13 antibody was omitted. Magnification X100.

Fig.94(a) **Sections of biopsy samples taken from human gastric mucosa**



Taken from the gastric antrum of a patient (2299) with *H. pylori*-negative chronic reactive gastritis. Immunostaining with anti-human BCA-1/CXCL13. Magnification X100. Strong positive staining for BCA-1/CXCL13 was observed in the gastric epithelial cells in the middle field of the photograph (see arrows). Some positive staining for BCA-1/CXCL13 was also observed in the inflammatory cells in the lamina propria.

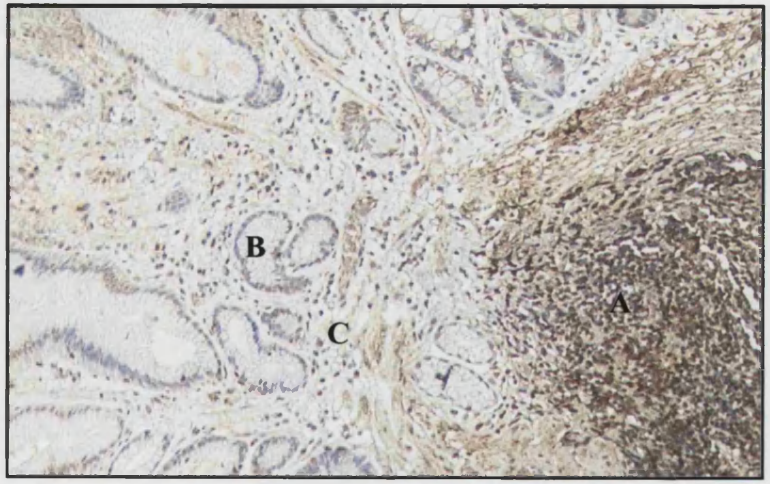
Fig.94(b)



Taken from the gastric antrum of a patient (2299) with *H. pylori*-negative chronic reactive gastritis. Negative control for Fig.94(a). No staining was observed when the primary anti-human antibody for BCA-1/CXCL13 was omitted. Magnification X100.

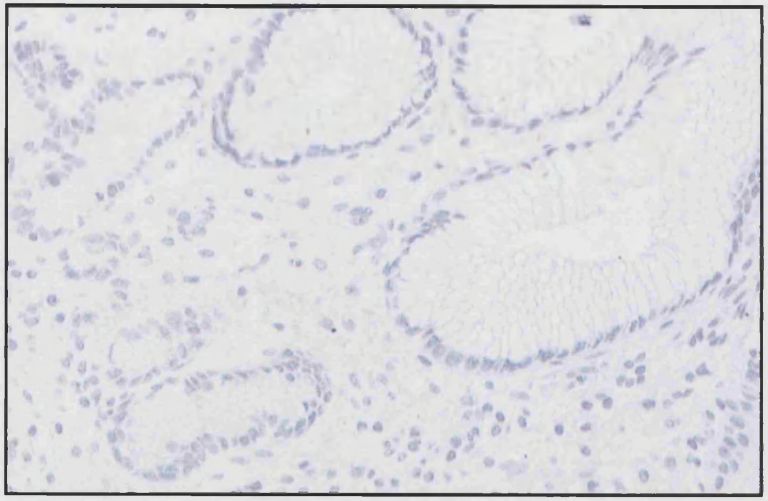
Sections of biopsy samples taken from human gastric mucosa

Fig.95(a)



Taken from a patient with 'MALT'oma (MALT lymphoma). Immunostaining with anti-human BCA-1/CXCL13 antibody. Magnification X100. Very heavy positive staining for BCA-1/CXCL13 in the area of the tumour on the right side of the photograph (marked A). The normal gastric epithelial cells are seen on the left of the photograph (marked B). In the middle of the photograph is an area of abnormal gastric epithelial cells (marked C). Some positive staining for BCA-1/CXCL13 can be seen in the lamina propria area.

Fig.95(b)



Taken from a patient with 'MALT'oma (MALT lymphoma). Negative control for Fig.95(a). No staining was seen when the primary anti-human BCA-1/CXCL13 was omitted. Magnification X200.

5.0 DISCUSSION

Gastric epithelial cells are important as they are the first-line cells in contact with ingested pathogens in the stomach. As they are involved with the body's defence in the stomach, their response against infections makes an interesting study. Gastric epithelial cells express chemokines which recruit specific leukocyte populations to the site of infection and induce inflammation of the stomach. Inflammation in the stomach is a precursor to other pathological conditions such as gastric ulcers, gastritis, gastric carcinoma and MALT-lymphoma.

Characterization of the kinetics of IL-8/CXCL8 and RANTES/CCL5 expression and production in gastric epithelial cell lines

This study involved two gastric epithelial cell lines, the AGS and MKN45 cell lines which were derived from human adenocarcinomas. They were used as gastric epithelial cell models as they had been well-established and have been used by many other researchers who implied they are suitable models of real gastric epithelial cells. They were also easy to maintain in culture, being adherent and immortal cell lines. Gastric cell lines were easy to use compared to real gastric epithelial cells which apoptosed rapidly in culture easily and could only be isolated in small numbers from gastric biopsies. Primary cultures of epithelial cells were also often contaminated with fibroblasts and other leukocytes from the biopsies which made it difficult to obtain results from a pure epithelial cell population. Cell lines are useful for obtaining detailed information about stimulation and regulation pathways but since the AGS and MKN45 cells used were derived from adenocarcinomas, there was still a possibility that they might behave in a different way compared to normal gastric epithelial cells.

In an *H. pylori* infection of real gastric epithelium, many types of leukocytes are recruited to the site of inflammation which involves chemokines from the 'CXC' and 'CC' families (Watanabe et al., 1997). Therefore, two chemokines were chosen for the purpose of this study, IL-8/CXCL8 from the 'CXC' chemokine family and RANTES/CCL5 from the 'CC' chemokine family. IL-8/CXCL8 has been shown to attract mainly neutrophils while RANTES/CCL5 attracts monocytes, lymphocytes,

basophils, eosinophils and T cells. A number of studies have shown that IL-8/CXCL8 and RANTES/CCL5 were expressed by inflamed gastric and intestinal mucosa. The pro-inflammatory cytokines IL-1α, TNF-α and IFN-γ, alone or in combination were used to stimulate the gastric cells to secrete chemokines (Strober, 1998). These cytokines are known to be expressed in response to *H. pylori* infection and have an adverse effect on the gastric mucosa (Crabtree et al., 1991; Moss et al., 1994; Noach et al., 1994; Fan et al., 1995; Peek et al., 1995 and Yamaoka et al., 1995). So far, the source of IL-1α, TNF-α and IFN-α which are increased in *H. pylori*-associated gastritis is unknown but it is hypothesised that activated macrophages may be the source of IL-1α and TNF-α (Sartor, 1994) while IFN-γ may be produced by natural killer cells or T lymphocytes (Karttunen et al., 1995). Pro-inflammatory cytokines are released in smaller amounts compared to chemokines when epithelial cells are in contact with pathogens but are potent cell activators. These pro-inflammatory cytokines can activate leukocytes, for example macrophages to mount inflammatory reactions to protect the host against the infection. An example of this mechanism *in vivo* in the gastric mucosa is as follows. IFN-γ which is expressed by intraepithelial T cells, found next to the epithelial cells on the basement membrane, up-regulates adhesion molecules on the epithelial cells. These adhesion molecules attract neutrophils and binds them with intercellular adhesion molecule-1 (ICAM-1) on to the apical part of the epithelial cells where they ingest pathogens.

IL-8/CXCL8 expression was studied because it is widely known that IL-8/CXCL8 mRNA, which is a 'CXC' chemokine is expressed when the gastric mucosa is infected with *H. pylori* and also it is very commonly expressed in inflammatory reactions in the stomach (Moss et al., 1994; Yamaoka et al., 1995; Yamaoka et al., 1996). IL-8/CXCL8 protein levels were also found to be raised with *H. pylori* infections (Crabtree et al., 1993; Noach et al., 1994; Gionchetti et al., 1994; Fan et al., 1995; Peek et al., 1995 and Yamaoka et al., 1997).

In our study, it was found that the AGS and MKN45 cell lines exhibited similar profiles of IL-8/CXCL8 mRNA expression. In Northern experiments, both cell lines did not demonstrate IL-8/CXCL8 mRNA expression when they were unstimulated. Interestingly, both the AGS and MKN45 cell lines were found to express the IL-

8/CXCL8 transcript in the RT-PCR experiments. This difference was since the RT-PCR experiments were more sensitive in detecting small amounts of IL-8/CXCL8 mRNA compared to the Northern analysis experiments. These RT-PCR results indicate that even in quiescent cells maintained in a 'defined' system in the absence of serum, low levels of IL-8/CXCL8 mRNA are expressed.

Stimulation of the AGS and MKN45 cells with IL-1α (3ng/ml) alone caused a rapid response of IL-8/CXCL8 mRNA expression within 1 hour of stimulation. This rapid IL-8/CXCL8 response has also been detected in the HT-29 colonic epithelial cell line (Kolios et al., 1996). In gastric epithelial cell lines, the IL-8/CXCL8 mRNA expression in response to IL-1α was short-lived, it was only detected for about 1 hour. TNF-α also caused a rapid response within 1 hour, but the mRNA expression was prolonged up to 12 hours in the AGS and MKN45 cells. This suggested that in gastric epithelial cells, TNF-α was a stronger pro-inflammatory stimulus for IL-8/CXCL8 expression than IL-1α.

In synovial fibroblasts and human vascular smooth muscle cells, IL-1 was a more potent stimulator of IL-8/CXCL8 mRNA than TNF-α (Jordan et al., 1996; Jordan et al., 1997). In these cells, peak expression was also later (4-6 hours) than the rapid 1 hour response detected in gastric cell lines. Is is thought that IL-8/CXCL8 expression in gastric carcinoma cell lines involves protein kinase C and tyrosine kinase activity (Beales et al., 1996).

When added simultaneously, IL-1α and TNF-α were additive and increased and prolonged the expression of IL-8/CXCL8 mRNA compared to the levels expressed by either one of the cytokines alone. These results are similar to the results in the HT-29 cells in a study conducted by Kolios et al.(1999). In the gastric mucosa, *in vivo* the presence of both cytokines would induce more gastric mucosa inflammatory damage compared to the effect from just one of the cytokines. It has been suggested that IL-1α and TNF-α may be expressed by a common pathway as their expression profile in intestinal epithelium was nearly identical to each other and they are both produced in response to the same stimuli and that they may compliment each other in inflammatory processes (Warhurst et al., 1998).

The cytokine IFN-γ (100U/ml) on its own did not induce the expression of IL-8/CXCL8 mRNA in either the AGS or MKN45 cell lines, this is similar to the results previously reported in the HT-29 colonic epithelial cells (Kolios et al., 1996). Similar results were also observed in the HT-29-19A cells, which are intestinal crypt cells and the intestinal villus absorptive cells, Caco-2 (Warhurst et al., 1998). This experiment demonstrated that IFN-γ did not play a role in initiating the expression of IL-8/CXCL8. Similarly, when IFN-γ (100U/ml) was added concurrently with IL-1α (10ng/ml) or TNF-α, the IL-8/CXCL8 mRNA expression was not modulated significantly from the profile obtained when IL-1α and TNF-α were added individually. However, in combination, (the cytomix combination of IL-1α, TNF-α and IFN-γ), there was generally a small prolongation of IL-8/CXCL8 mRNA expression.

The lack of response to IFN-γ in gastric epithelial cell lines emphasises a difference from colonic epithelial cells (HT-29) where the addition of cytomix increased the expression of IL-8/CXCL8 mRNA above that of IL-1α and TNF-α added together without IFN-γ (Kolios et al.,1996). IFN-γ belongs to the Th1 type of response which usually is involved with the initiation of inflammatory responses unlike the Th2 type of response which is more anti-inflammatory in some cell lines (Kolios et al., 1996).

Expression of RANTES/CCL5 mRNA, a 'CC' chemokine was also studied in both the AGS and MKN45 cell lines. Unstimulated cells did not express RANTES/CCL5 mRNA constitutively. A difference was observed between the AGS and the MKN45 cells, since under all the stimuli conditions tested, RANTES/CCL5 mRNA expression was not induced in the AGS cells. Interestingly, the stimuli needed to induce the expression of RANTES/CCL5 mRNA in the MKN45 cell line was different from that required to induce IL-8/CXCL8 mRNA. The minimum stimuli which could induce the expression of RANTES/CCL5 mRNA were a combination of TNF-α (30ng/ml) and IFN-γ (100U/ml). Since TNF-α alone did not induce RANTES/CCL5, IFN-γ was important in activating the appropriate signalling pathway not required in gastric cells for IL-8/CXCL8 production. This synergistic effect of TNF-α and IFN-γ in expressing RANTES/CCL5 had previously been found in fibroblasts (Rathanaswami et al., 1993), endothelial cells (Marfaing-Koka et al., 1995), bronchial epithelial cells (Berkman et al., 1995; Stellato et al., 1995) and HT-

29 cells (Kolios et al., 1999). In the current study, the addition of IL-1α did not significantly modulate RANTES/CCL5 expression. This suggests that the signalling pathways activated may vary in different cell types, since low levels of RANTES/CCL5 have been shown to be expressed in vascular smooth muscle cells in response to IL-1α alone (Jordan et al., 1997).

The profile of RANTES/CCL5 mRNA expression in MKN45 cells was different from IL-8/CXCL8 mRNA since the onset of RANTES/CCL5 mRNA was later, it was first expressed at 6 hours post-stimulation with a peak at 12 hours post-stimulation. The late expression of RANTES/CCL5 mRNA at 24 hours has previously been reported in HT-29 cells (Kolios et al., 1996), human airway (John et al., 1997) and vascular smooth muscle cells (Jordan et al., 1997). It has previously been suggested that the late onset of RANTES/CCL5 may be due to the requirement of an unknown intermediary protein to initiate RANTES/CCL5 mRNA expression (Rathanaswami et al., 1993).

A possible explanation for this patterned release of chemokines, IL-8/CXCL8 (early) and RANTES/CCL5 (late) may play a role in mediating the accumulation of specific leukocyte populations at different phases of the inflammatory response (Strober, 1998).

Interestingly, even though both the AGS and MKN45 epithelial cell lines were derived from gastric adenocarcinomas, they both behaved similarly in IL-8/CXCL8 mRNA expression but differently with respect to RANTES/CCL5 production. There were also differences in the other types of chemokines which were detected to be expressed by these two cell lines. The differences could be due to these cell lines being derived from different individuals and possibly from different stages of cancer type and development.

ELISA analysis of the AGS and MKN45 cell supernatants indicated that unstimulated quiescent (serum free) cells did not secrete IL-8/CXCL8 or RANTES/CCL5 protein. The concentration of IL-8/CXCL8 protein secreted into the medium increased in a time-dependent manner (1-24 hours) when the AGS and MKN45 cells were stimulated with IL-1α, TNF-α or cytomix indicating that mRNA

was translated into secreted protein, and that the protein was still produced even once the short-lived mRNA was no longer detectable.

In the AGS cells in response to all stimuli, IL-8/CXCL8 increased steadily in the medium until 48 hours. Increases were not detected from 48-72 hours for two possible reasons, mRNA was expressed rapidly (1 hour) and was short-lived, hence its translation into protein decreased after 48 hours. Since these experiments were performed in a 'defined' serum-free medium, beyond 48 hours in the absence of serum, the cells were probably starting to enter apoptosis, although the viability assays did not indicate any significant decrease in viability up to 72 hours.

Protein secretion indicated that in the AGS cells, IL-1α and TNF-α stimuli induced a similar IL-8/CXCL8 protein secretion, even though IL-1α induced less detectable mRNA than TNF-α. IL-1α and TNF-α added simultaneously induced approximately twice the amount of IL-8/CXCL8 compared to individual responses indicating responses to these stimuli were additive.

The amount of IL-8/CXCL8 secreted after 48 hours of maximum stimulation with cytomix in the AGS cells was approximately 8ng/ml, this was a similar concentration to the amount secreted after 48 hours in the MKN45 cells.

In the MKN45 cells, the IL-8/CXCL8 mRNA induced by IL-1α was not translated into high levels of secreted IL-8/CXCL8 protein, indicating that in contrast to the AGS cells, TNF-α was a more potent inducer of secreted IL-8/CXCL8 in the MKN45 cells.

ELISA analysis demonstrated that RANTES/CCL5 protein was secreted by the MKN45 cells stimulated with TNF-α and IFN-γ, with similar results when IL-1α was added simultaneously. Secreted RANTES/CCL5 remained low until 6 hours post-stimulation and a late expression occurred since the RANTES/CCL5 mRNA was not detected until 6 hours. The concentration of RANTES/CCL5 protein secreted at 24 hours was 3ng/ml, this was lower than the peak levels of IL-8/CXCL8 secreted by the MKN45 cells over 24 hours.

RANTES/CCL5 continued to be secreted as protein even after the mRNA levels had decreased. The kinetics of TNF-α and IFN-γ induced RANTES/CCL5 secretion

suggest that this chemokine may be involved in the recruitment and activation of T cells over long periods.

Regulation of IL-8/CXCL8 and RANTES/CCL5 expression and production by IL-4 and IL-13

Previous studies have indicated that gastric epithelial cell lines demonstrated a Th1 type of reaction in response to pro-inflammatory mediators, such as cytokines or *H. pylori*. In the current study, the novel immunomodulatory effects of the Th2 cytokines, IL-4 and IL-13 on gastric epithelial cell line chemokine expression were examined. These cytokines did not always behave in the same way in regulating chemokine expression in every cell line.

The cells were stimulated with higher concentrations of IL-1α (10ng/ml), TNF-α (100ng/ml) and IFN-γ (300U/ml) ('cytomix') to obtain maximal stimulation of these cell lines and high expression of IL-8/CXCL8 and RANTES/CCL5 protein in the AGS and MKN45 cell lines. These high concentrations for the cytokines had previously been used in our laboratory on the HT-29 cell line to induce maximum expression of IL-8/CXCL8, MCP-1/CCL2 and RANTES/CCL5 mRNA and protein (Kolios et al., 1996).

Pre-stimulation with IL-4 did not have any significant effect on the production of IL-8/CXCL8 protein or mRNA in the AGS or MKN45 cell lines when they were stimulated with 'cytomix'. Therefore, IL-4 did not act as a stimulating or inhibitory cytokine on the production of IL-8/CXCL8 protein in either cell line. Pre-stimulation with IL-13 also had no significant effect on the IL-8/CXCL8 protein secretion by the AGS cells or IL-8/CXCL8 mRNA and protein expression in the MKN45 cells. Similar to the results obtained by Kolios et al. (1996), when HT-29 cells were stimulated with TNF-α and IFN-γ, IL-4 and IL-13 had no effect on IL-8/CXCL8 production.

A differential effect of IL-4 and IL-13 on IL-8/CXCL8 and RANTES/CCL5 chemokine expression was demonstrated, since IL-4 and IL-13 were both able to inhibit RANTES/CCL5 protein production in a concentration-dependent way in the MKN45 cell line. The Northern analysis experiment indicated that the mechanism of action was by the down-regulation of mRNA expression.

Both of these results were similar to the effects reported by Kolios et al., (1997) in the HT-29 colonic epithelial cell line where the 'CC' chemokines MCP-1/CCL2 and RANTES/CCL5 were inhibited by IL-4 and IL-13 whereas IL-8/CXCL8 was not affected. RANTES/CCL5 induced by a combination of TNF-α and IFN-γ has also been inhibited by IL-13 in endothelial cells (Marfaing-Koka et al., 1995) and human airway smooth muscle cells (John et al., 1997). Responses may be cell-dependent since IL-13 did not affect the secretion of RANTES/CCL5 protein in smooth muscle cells (Jordan et al., 1997) and in human airway epithelial cells (Berkman et al., 1996).

These results indicate that both the MKN45 gastric epithelial cells and HT-29 colonic epithelial cells have functional IL-4 and IL-13 receptors. IL-4 and IL-13 receptors are closely related but are distinct. These receptors are known to have independent biological functions; although RANTES/CCL5 down-regulation in the MKN45 cells was a similar response to both IL-4 and IL-13.

The ability to inhibit RANTES/CCL5 but not IL-8/CXCL8 production indicates that differential signalling pathways by TNF-α and IFN-γ are used by these two chemokines and that in the MKN45 and HT-29 cells, there is no common inhibitory target for IL-4 and IL-13. The ability of Th2 cytokines to inhibit RANTES/CCL5 *in vivo* may play an important role in modulating the inflammatory process by inhibiting T cell monocyte and eosinophil recruitment into the gastric epithelium. IL-4 and IL-13 may be able to decrease inflammation associated with infection with *H. pylori* and other monocyte and macrophage derived cytokines and chemokines (Minty et al., 1993).

In vivo, it is known that chemokine regulation is complex. In a separate study by Kolios et al. (1996; 1999), it has been shown that stimulation of HT-29 cells with TNF-α alone induced IL-8/CXCL8 production which was not inhibited by IL-13. This suggests that it is signalling by IFN-γ (when TNF-α and IFN-γ are added in combination), which enables the IL-8/CXCL8 production to be inhibited by IL-13. In HT-29 cells (Kolios et al., 1996) and vascular smooth muscle cells (Jordan et al., 1997), stimulation of IL-8/CXCL8 production by IL-1α alone showed a significant increase by IL-13. Therefore, IL-13 has a bifunctional role, depending on the type of stimulus used.

It has been suggested that the anti-inflammatory effect of IL-13 could be due to the enhancement of the IL-1 receptor antagonist (IL-ra) which blocks the IL-1 signalling and results in a direct inhibition of chemokine transcription (Muzio et al., 1994). However, this theory cannot explain the increase in IL-1 induced IL-8/CXCL8 detected in the HT-29 (Kolios et al., 1996) and vascular smooth muscle cells (Jordan et al., 1997). Since RANTES/CCL5 production in the MKN45 cells is not stimulated by IL-1, the inhibition of cytomix induced RANTES/CCL5 by IL-4 and IL-13 cannot be due to the enhancement of the IL-1ra.

In conclusion, the responses measured as a result of IL-4 and IL-13 modulation are variable depending on the cell type studied, the stimulus used and the chemokine expression investigated. The inability of IL-4 and IL-13 to increase or decrease IL-8/CXCL8 production in cytomix stimulated gastric epithelial cells suggests that these Th2 cytokines may not modulate neutrophil infiltration *in vivo*.

Chemokine expression in gastric epithelial cells

Chemokine expression has been widely studied in leucocytes where they are involved in chemotaxis. Chemokines have also been shown to be produced by 'non-immune'/haematopoietic cells such as epithelial cells, endothelial cells, smooth muscle cells, mesangial cells and others. This expression may be important in attracting inflammatory cells into the extravascular tissue site. Some chemokine receptors had also been found to be expressed by normal tissue ('non-immune' cells) on these cells suggesting that they may also be able to respond to stimulation with certain chemokines which may modulate cell functions and behaviour.

In this study, it has been shown by Northern analysis and RT-PCR that gastric epithelial cell lines express the chemokines IL-8/CXCL8 and RANTES/CCL5 when stimulated by pro-inflammatory cytokines, with RANTES/CCL5 showing later expression. Using RT-PCR, but not Northern analysis, IL-8/CXCL8 mRNA could be detected in unstimulated cells suggesting that IL-8/CXCL8 is expressed by epithelial cells at low levels constitutively. IL-8/CXCL8 expression in gastric epithelium has been reported to be expressed by unstimulated and increased in pro-inflammatory cytokine-stimulated AGS cells (Beales et al., 1997; Nakachi et al., 2000) and

MKN45 epithelial cells (Aihara et al. 1997, Sharma et al.,1995; Yasumoto et al., 1996.).

In addition to IL-8/CXCL8, the current study has also shown that other CXC chemokines including MIG/CXCL9 and IP-10/CXCL10 are expressed in gastric epithelial cells in response to pro-inflammatory cytokines. Both of these CXC (ELR negative) chemokines are chemoattractants for activated T cells.

Prior to this, IP-10/CXCL10 has been associated with psoriasis. Eck et al. (2000) found that in *H. pylori* gastritis, MIG/CXCL9 and IP-10/CXCL10 were expressed by endothelial cells and mononuclear cells in the lamina propria, at the sites of T cell infiltration.

MIG/CXCL9 was also expressed constitutively in unstimulated AGS and MKN45 cells, therefore, may play a role in maintaining 'normal' gastric epithelial cell function. MIG/CXCL9 has been implicated in regulating the homing of intraepithelial lymphocytes (IEL) in the host gastric mucosa.

'CC' chemokines for example the monocyte chemoattractant proteins, MCP-1/CCL2 and MCP-3/CCL7 and LARC/CCL20 in addition to RANTES/CCL5 have also been shown to be increased in expression in gastric epithelial cells stimulated with pro-inflammatory cytokines. MCP-1/CCL2 has been implicated in a number of diseases including rheumatoid arthritis, inflammatory heart disease, granulomatous disease, bone trauma and atherosclerosis. MCP-1/CCL2 has also been found to be induced in *H. pylori* gastritis (Shimoyama et al., 1998; Mori et al., 2001).

The chemokine LARC/CCL20 has not been previously reported to be expressed in the gastric epithelium. It is a known monocyte/T cell chemoattractant and the expression has been shown to be increased to high levels by pro-inflammatory cytokines. These results suggest that it is an important chemokine in gastric inflammation. In the AGS and MKN45 cells, LARC/CCL20 mRNA was also expressed at high levels constitutively, since it was detected in unstimulated cells and the very high levels stayed the same throughout the stimulation with 'cytomix' indicating that LARC/CCL20 may play a role in 'normal' homeostasis. LARC/CCL20 was shown for the first time in our study to be expressed by gastric epithelial cells and this implied that gastric epithelial cells behaved in a similar way

to mouse intestinal epithelial cells (Tanaka et al., 2000) and human colonic epithelial cells (Izadpanah et al., 2001). The probable function of LARC/CCL20 is to attract lymphocytes and dendritic cells towards the epithelial cells for immunosurveillance when the epithelial cells were not stimulated. LARC/CCL20 may also be involved in inflammatory processes when the epithelial cells are stimulated with 'cytomix'.

Chemokine receptor expression by gastric epithelial cells

RT-PCR analysis was included in this study to provide information about the chemokine receptors expressed by gastric epithelial cells. Although there have been many studies published about receptor expression on leukocytes, there is less known about receptor expression on 'normal tissue' cells. To date, there has been no studies demonstrating receptor expression on gastric epithelial cells. It is generally accepted that only low levels of most chemokine receptors are expressed by normal tissue ('non-immune' cells). In the current study, most chemokine receptors except CXCR4 were not expressed at high enough levels to be reproducibly detected in unstimulated AGS or MKN45 cells. The RT-PCR technique used, although highly sensitive and specific, sometimes gives variable accuracy. It is dependent on several factors such as the choice of primers, the choice of target DNA, preparation of the specimen and technical issues pertaining to the experimental procedure (Dunn et al., 1997). The expression of CXCR4 obtained in the MKN45 cells is comparable to the results reported on the HT-29 colonic epithelial cells, where no receptors except CXCR4 were detected (Jordan et al., 1999). Dwinell et al. (1999) showed that CXCR4 and CCR5 were expressed in all human intestinal epithelial cell lines investigated. Other chemokine receptors were only detected in some of the cell lines suggesting variations in different cells. Although CXCR4 was expressed by the MKN45 gastric epithelial cells, this receptor was not expressed by the AGS cells. This proved that although there was a lot of homology between the AGS and MKN45 cell lines, there were still differences between each cell line. Other receptors expressed faintly by the MKN45 cells were CCR2 and CXCR5, while the AGS cells expressed CCR1, CCR2 and CXCR1. CXCR4 has also been found on endothelial cells (Feil et al., 1998) and astrocytes (Tanabe et al., 1997).

SDF-1α/CXCL12, which is the only ligand for CXCR4, was not expressed by the AGS and MKN45 cell lines as shown by RT-PCR experiments. Shibuta et al. (1997)

also found that SDF-1α/CXCL12 was not expressed in a premalignant colonic adenocarcinoma. This result was also found in the HT-29 cells by Jordan et al. (1999). However, Agace et al. (2000) found that SDF-1α/CXCL12 mRNA was found to be expressed constitutively in normal small intestine epithelial cells.

Therefore, CXCR4 cannot be bound in these epithelial cells by an autocrine stimulation. Since SDF-1α/CXCL12 is known to be expressed by fibroblasts (Tashiro et al., 1993) and has also been detected in normal gastric tissue (Shibuta et al., 1997), CXCR4 may be activated by paracrine stimulation.

SDF-1α/CXCL12 and CXCR4 is a unique chemokine-receptor ligand pair as CXCR4 is one of only a few chemokine receptors which has only one identified specific ligand. SDF-1α/CXCL12 plays a fundamental role in development, in the formation of the vasculature of the gastrointestinal tract (Tachibana et al., 1998). In our study, one normal patient was shown to express SDF-1α/CXCL12 and CXCR4 constitutively by RT-PCR, this result is parallel to the findings of Jordan et al. (1999) who found that SDF-1α/CXCL12 was expressed constitutively in many tissues including the colon. Since both SDF-1α/CXCL12 and CXCR4 can be detected in the gastric epithelium constitutively, this suggests that there is a physiological role for the receptor in normal gastric homeostasis. SDF-1α/CXCL12 may have a role in immune surveillance where it recruits T cells and monocytes.

The role of CXCR4 and SDF-1α/CXCL12 in normal gastric epithelium is unclear, Since it is expressed in epithelial cells which normally undergoes rapid cell division and maturation, this may suggest a role for SDF-1α/CXCL12 and CXCR4 in the renewal and maintenance of epithelium (Jordan et al., 1999). SDF-1α also plays a role in carcinogenesis as a tumour suppressor gene, by inhibiting tumour vascularization (Shibuta et al., 1997), which is important in many digestive tract cancers including primary colon cancers and hepatomas. This report also suggests that unusually in gastric cancer, there is no correlation between gastric cancer and clinico-pathological characteristics.

Although in colonic epithelium and other tissues, CXCR4 may mediate HIV (Feng et al., 1996; Endres et al., 1996) infection, however the role of CXCR4 in mediating HIV infections in the stomach is presently uncertain. CXCR4 might play a role for

the entry of this virus into the human via the gastrointestinal tract. Similarly, if anti-CXCR4 drugs could be designed in the future, HIV entry into the host cells could be blocked.

In gastric epithelial cells, for example the MKN45 cell line as shown by RT-PCR and the epithelial cells as shown by immunohistochemical staining of the gastric biopsies, CXCR4 was expressed at high levels, other chemokine receptors were also detected but appeared to have very low or variable expression.

Interestingly, pro-inflammatory cytokines such as IL-1β and TNF-α have been shown to down-regulate the expression of CXCR4 on human umbilical vein endothelial cells (HUVEC) and then up-regulating it (Gupta et al., 1998). In the same study, however IFN-γ was found to down-regulate the expression of CXCR4 on the endothelial cells.

Even though the AGS and MKN45 cell lines expressed MIG/CXCL9 constitutively, both cell lines did not express the chemokine receptor for MIG/CXCL9 , which was CXCR3. The absence of this chemokine receptor implies that MIG/CXCL9 did not have an autocrine effect on the gastric epithelial cells.

CXCR1 and CXCR5 were expressed constitutively and were up-regulated by pro-inflammatory cytokines. In our RT-PCR experiments, this was demonstrated by the AGS cells which expressed constitutive CXCR1 receptor faintly, and this expression was enhanced when the AGS cells were stimulated with 'cytomix' for the duration of 1, 3 and 12 hours. The major ligand for CXCR1 is IL-8/CXCL8 and the only CXCR5 ligand is BCA-1/CXCL13. Both of these chemokines have been shown to be up-regulated in gastric epithelium by gastritis. CXCR1 expression was not detected in colonic epithelial cells (Dwinell et al., 1999) and has not previously been investigated in gastric epithelial cells. Expression of CXCR1 by human microvascular endothelial cells (Salcedo et al., 2000) may be important in angiogenesis. The role of CXCR1 in epithelial cells is unknown, but may be important in inflammatory responses since in the AGS cells it was up-regulated by pro-inflammatory cytokines.

CXCR5 expression has not been previously reported in gastric epithelial cells. It is known to be expressed by B and T lymphocytes (Legler et al., 1998; Zlotnik et al.,

1999) and has previously been detected in the stomach (Gunn et al., 1998). It is also known to be a specific co-receptor for HIV-2 (Kanabe et al., 1999). It's role in gastric epithelial cell homeostasis is unknown, but our immunohistochemical studies have shown that BCA-1/CXCL13 is expressed by 'normal' gastric tissue, therefore paracrine stimulation of epithelial cells through the CXCR5 receptor is possible. BCA-1/CXCL13 was not expressed by the MKN45 cells.

CCR1 and CCR2, the receptors for MCP-1/CCL2 and MCP-3/CCL7 and RANTES/CCL5 were also expressed at very low levels by some gastric epithelial cell lines. In our study, the unstimulated AGS cells expressed the CCR1 chemokine receptor and this receptor appeared to have been down-regulated after the cells had been stimulated with 'cytomix'. This is similar to some other studies which reported that pro-inflammatory cytokines could also down-regulate the expression of chemokine. All of their ligands were shown to be expressed by stimulated gastric epithelial cells, therefore autocrine responses are possible. The AGS cells in our study expressed both CCR2 and the ligand for CCR2 which was MCP-1/CCL2. Interestingly, CCR2, was also found on the MKN45 cells which expressed MCP-1/CCL2 constitutively as well as during stimulation with 'cytomix' for 1, 3 and 12 hours. Therefore, MCP-1/CCL2 may have autocrine regulatory effects on gastric epithelial cells which express the CCR2 chemokine receptor. However, the AGS or MKN45 cell line did not express any BCA-1/CXCL13 at all, which is the corresponding ligand for the CXCR5 receptor.

Both of these receptors, CCR1 and CCR2, have been reported to be expressed by colonic epithelial cells (Dwinell, et al., 1999). They have not previously been reported in gastric epithelium. Expression of CCR1 and CCR2 have also been reported in human vascular smooth muscle cells (Hayes et al., 1998) and it has been speculated that responses via these receptors may stimulate smooth muscle cell regrowth and migration (Yue et al., 1994). It is possible that BCA-1/CXCL13 may stimulate 'normal' gastric epithelium to divide/proliferate but this needs to be investigated in further studies.

Chemokine expression by gastric mucosal biopsies

Chemokines have been shown in numerous studies to be important in the pathogenesis of various inflammatory type-diseases including atherosclerosis (MCP-1/CCL2)(Yla-Herttuala et al., 1991), asthma (RANTES/CCL5)(Stellato et al., 1995); (eotaxin/CCL11)(Lamkhioued et al., 1997), psoriasis (MIG/CXCL9)(Goebeler et al., 1998), psoriasis (IP-10/CXCL10) and arthritis (MCP-1/CCL2)(Koch et al., 1992). A number of 'CXC' chemokines (GRO-α/CXCL1 (Kusugami et al., 1997), ENA/CXCL5 (Rieder et al., 1997), IL-8/CXCL8 (Ishihara et al., 1996; Martin-Guerrero et al., 2000), MIG/CXCL9 (Eck et al., 2000), IP-10/CXCL10 (Eck et al., 2000) and 'CC' chemokines MCP-1/CCL2 (Mori et al., 2001), MIP-1α/CCL3 (Yamaoka et al., 1998) and RANTES/CCL5 (Yamaoka et al., 1998) have previously been shown to be involved in gastritis.

In the current study, chemokine expression in mucosal gastric biopsies enabled a comparison between 'normal' patients, patients with *H. pylori*-positive gastritis and patients with *H. pylori*-negative gastritis. In the latter patient group, there is a possibility that dietary components or drugs acted as antigens when ingested (Yamaoka et al., 1998).

The biopsies examined in this study were from the mucosa and therefore included a number of cell types. In addition to epithelial cells, samples included smooth muscle cells, fibroblasts, endothelial cells and especially in the inflamed samples, numerous leukocytes were also present. Comparison of the results obtained by RT-PCR analysis of gastric biopsies and cultured gastric epithelial cell lines has enabled the data to be assessed regarding the possibility that 'real' epithelial cells in biopsies may be contributing to the expression of individual chemokines. This information would be enhanced if analysis was performed on gastric epithelial cells purified from biopsy material. The poor viability and apoptosis previously reported (Ohsuga et al., 2000) prevented this analysis in the current study. Future studies may be improved if human gastric epithelial cells could be isolated from large gastric surgical specimens, but such samples are not easily obtained.

Gastric biopsies from patients without gastritis did not express IL-8/CXCL8 mRNA, suggesting this chemokine is not especially important in normal gastric mucosal homeostasis. IL-8/CXCL8 expression was detected in some patients with gastritis.

IL-8/CXCL8 expression increases in gastritis and *H. pylori* infection (Crabtree et al., 1994; Noach et al., 1994; Ishihara et al., 1996; Katagiri et al., 1997; Yamaoka et al., 1998). Even though *H. pylori* is non-invasive of the epithelial cells it causes gastric mucosal damage. The presence of *H. pylori* infection can also stimulate the leukocyte production of pro-inflammatory cytokines which induce production of chemokines, such as IL-8/CXCL8 by the epithelial cells. Pathogenic factors produced by the *H. pylori* have also been shown to directly stimulate IL-8/CXCL8 production by gastric epithelial cells (McGee et al., 2000). Increased IL-8/CXCL8 production attracts more neutrophils to infiltrate the gastric epithelium, helping to eliminate the *H. pylori* infection but it can also cause additional gastric mucosal damage (Martin-Guerrero et al., 2000).

IL-8/CXCL8 is known to be expressed by epithelial cells, endothelial cells, monocytes and fibroblasts (Baggiolini et al., 1989). However, in organ cultures of gastric mucosal tissues, epithelial cells were shown to be the main producers of IL-8/CXCL8 and GRO-α/CXCL1 (Ohsuga et al., 2000), therefore in the gastric biopsies in the current study, the epithelial cells are most likely to be the main producers of IL-8/CXCL8 (Martin-Guerrero et al., 2000). Other cells might be expressing IL-8/CXCL8 at lower levels. IL-8/CXCL8 is known to attract neutrophils to the site of infection. In addition, IL-8/CXCL8 and other 'CXC' chemokines which contain the 'ELR' motif demonstrate the ability to induce angiogenesis via proliferation of endothelial cells (Gale et al., 1999). Angiogenesis is important in tissue repair and the development such as in the increased growth and metastasis of gastric cancers. In a study by Wang et al. (1996), IL-8/CXCL8 had been implicated in the development of non-small cell lung cancer via the angiogenesis process.

Other 'CXC' chemokines including MIG/CXCL9 and IP-10/CXCL10 were not expressed in detectable levels in normal gastric mucosa. Expression of both was increased in patients with gastritis. Expression was highest in patients with gastritis but with no *H. pylori* infection. In biopsies taken from the antrum, 100% of the

patients expressed MIG/CXCL9 and 50% expressed IP-10/CXCL10, compared to only 16% of patients infected with *H. pylori* who expressed both MIG/CXCL9 and IP-10/CXCL10. These results strongly suggest that MIG/CXCL9 is an important chemokine in inflammation in gastric tissue. MIG/CXCL9 and IP-10/CXCL10 act synergistically to recruit T cells in *H. pylori*-associated gastritis (Eck et al., 2000).

Expression of MIG/CXCL9 and IP-10/CXCL10 demonstrate that a Th1 reaction occurs in response to *H. pylori* infection (Sallusto et al., 1998(b)). These chemokines can be produced in gastric tissue and induce responses in cells expressing CXCR3, which includes Th1 cell types. As Th1 reactions are not adequate to eradicate extracellular bacterial infections such as *H. pylori* infections, it has been implied that this Th1 reaction has a pathogenic rather than a protective role towards the gastric mucosa (D'Elios et al., 1997; Bamford et al., 1998). Results in the current study suggest that MIG/CXCL9 and IP-10/CXCL10 are not only involved in *H. pylori* induced gastritis, but are equally important in gastritis induced by other unknown factors.

Unlike IL-8/CXCL8, MIG/CXCL9 and IP-10/CXCL10 chemokines, both of these 'non-ELR'-containing' 'CXC' chemokines are important for the angiostatic process in the stomach (Strieter et al., 1995). Angiostasis may inhibit the growth of gastric tumours in the stomach (Moore et al., 1998). Therefore, MIG/CXCL9 and IP-10/CXCL10 are important as they act opposite to the effects of the angiogenic 'ELR'-containing 'CXC' chemokines chemokines GRO-α/CXCL1 and IL-8/CXCL8, therefore maintaining a fine balance between angiogenic and angiostatic processes in the stomach. The presence of MIG/CXCL9 and IP-10/CXCL10 limit the damage achieved by the other angiogenic stimuli, such as IL-8/CXCL8.

The cell source of MIG/CXCL9 and IP-10/CXCL10 expression in gastric biopsies is unknown. Other reports have found that MIG/CXCL9 and IP-10/CXCL10 were not expressed by gastric epithelial cells but were expressed by the endothelial cells in the small blood vessels in the lamina propria and by mononuclear cells where there are T cell infiltrations (Eck et al., 2000). RT-PCR analysis in this study have shown that cytokine stimulated AGS and MKN45 cells express MIG/CXCL9 and IP-10/CXCL10. Bronchial epithelial cells produce MIG/CXCL9 and IP-10/CXCL10

and it is known that epithelial cells can express these chemokines when infected with tuberculosis (Sauty et al., 1999).

The 'CXC' chemokine SDF-1α/CXCL12 was expressed by some patients in all disease categories, it was also detected in some samples from 'normal' patients. The inability to induce SDF-1α/CXCL12 in the AGS and MKN45 cells suggests that it was not secreted by epithelial cells. Fibroblasts that are present in gastric biopsies are a probable source of SDF-1α/CXCL12 expression (Jordan et al., 1999). The detection of SDF-1α/CXCL12 in non-gastritis patients suggests it may play a role with the CXCR4 receptor in normal gastric homeostasis. SDF-1α/CXCL12 is known to be important in the development of the heart (cardiogenesis) and haematopoiesis and nothing is known about its role in the stomach apart from encouraging vascularization of the stomach by angiogenesis. Another possible function of the SDF-1α/CXCL12 chemokine was in immune surveillance where SDF-1α/CXCL12 had been shown to recruit resting T lymphocytes, monocytes and intraepithelial T cells in the gut. SDF-1α/CXCL12 expression was not increased in gastritis suggesting it did not play an important role in inflammation. *H. pylori* also did not have an effect on the expression of SDF-1α/CXCL12.

MCP-1/CCL2 has been shown to be expressed by many types of cells such as monocytes, macrophages, fibroblasts and epithelial cells but in the stomach, epithelial cells may be the biggest producers of MCP-1/CCL2 (Watanabe et al., 1997). MCP-1/CCL2 was detected in samples from gastric body (100%) and antrum (50%) in normal patients suggesting it may play a role in normal cell behaviour. MCP-1/CCL2 expression was similarly detected in patients with gastritis in the presence or absence of *H. pylori*. Mori et al. (2001) found that *H. pylori* could cause the expression of MCP-1/CCL2 mRNA and protein to be increased in gastric epithelial cells. Our result is concordant with the result of Watanabe et al. (1997) who showed that MCP-1/CCL2 was expressed in gastric biopsies independent of the presence of *H. pylori* and they suggested that MCP-1/CCL2 may have a physiologic role for it to be expressed constitutively. One possible role for MCP-1/CCL2 would be to stimulate angiogenesis in the stomach, although it may also have a pathological role in contributing to the aetiology of tumour formation (Salcedo et al., 2000).

MCP-1/CCL2 has also been shown to be elevated in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis. In these clinical conditions, MCP-1/CCL2 was responsible for attracting macrophages and eosinophils into the lamina propria, hence increasing the inflammatory reactions at the site of action (Reinecker et al., 1995). MCP-1/CCL2 may have the same role in gastritis since the expression of MCP-1/CCL2 is increased in gastritis and the strong detection indicated increased expression.

The 'CC' chemokines MIP-1α/CCL3 and RANTES/CCL5 were detected in more patients with gastritis than in gastric tissue from 'normal' patients. These results have been contradicted in a study by Yamaoka et al. (1998) who concluded that 'CXC' chemokine levels increased in *H. pylori* infection but concluded that 'CC' chemokines did not play a role in infiltration of monocytes in *H. pylori* infection. These chemokines are involved in attracting lymphocytes, monocytes and macrophages. In the current study, both chemokines were detected in the gastric body samples in more patients with gastritis without *H. pylori* infection, than in patients who were also infected with *H. pylori*. Interestingly, the main producers of MIP-1α/CCL3 were found to be macrophages and not gastric epithelial cells (Kusugami et al., 1999). MIP-1α/CCL3 protein levels were also found by Ando et al. (1998) to be mostly expressed in patients with gastric ulcers or duodenal ulcers compared to patients with erosive gastritis or normal patients.

In previous studies, elevated levels of MIP-1α/CCL3 mRNA and protein levels have been found to be increased in patients infected with *H. pylori* (Kusugami et al., 1999; Sato et al., 1999). It has been suggested that raised levels of these chemokines were used to mount an inflammatory response against *H. pylori*. Similarly, Yamaoka et al. (1998) found that MIP-1α/CCL3 mRNA levels were raised with *H. pylori* infection, however, the protein levels were below sensitivity levels in the experiment and no conclusions could be made. Separate studies have shown that MIP-1α/CCL3 protein levels were raised in parallel with positive *H. pylori* infection (Ando et al., 1998).

More association of MIP-1α/CCL3 expression with *H. pylori* infection may have been detected in the current study if the number of patients included had been

increased. Variation between individual patients can be expected since the length of time that each patient has been infected/suffered from gastritis will not be identical. Some patients will be in the early stages of an inflammatory response, whereas other patients may be at a later stage when cell infiltration is different.

RANTES/CCL5 mRNA was detected in patients with gastritis in the current study. It has previously been reported that RANTES/CCL5 mRNA is elevated in *H. pylori* infection but protein levels are not always increased. This is explained since *H. pylori* may interrupt the translation of RANTES/CCL5 mRNA into protein or the amount of RANTES/CCL5 mRNA was too little to be translated into protein (Yamaoka et al., 1998). In this study, there did not also seem to be any correlation between mononuclear cells and polymorphonuclear leucocytes with RANTES/CCL5 in *H. pylori* infections. More studies would have to be carried out to determine the role of RANTES in *H. pylori* infections especially since eradication of *H. pylori* did not demonstrate a decrease in RANTES/CCL5 expression. In the current study, more patients were demonstrated to have RANTES/CCL5 expression in the absence of *H. pylori* than those suffering from the infection. This suggests that *H. pylori* is less important in inducing RANTES/CCL5 than general unknown causes of gastritis.

In the current study, particularly evident from the detection of MIP-1α/CCL3 and RANTES/CCL5, it is evident that chemokines are detected in more patients in the antrum than in the body sections of the stomach. This suggests that gastritis might be more pronounced in the antrum (Queiroz et al., 1988; Stolte et al., 1990). In some cases, higher levels of expression (strong bands detected by RT-PCR) were also detected in the samples from the antrum (BCA-1/CXCL13 and MCP-1/CCL2).

Stolte et al (1990) found that the grade of antral gastritis correlated with the grade of *H. pylori* colonization. Possible explanations for more severe gastritis to be found in the antrum could be due to gastrin secretion in the antrum induced by *H. pylori* urease which increases the secretion of gastric acid and induce mucosal damage (Bayerdoffer et al., 1992) or decreased mucin production in the antrum as a result of *H. pylori* ammonia production which weakens the antral mucosal barrier (Tsuji et al., 1989). The antrum and gastric body could also exhibit different mucosal reactivity in response to *H. pylori*.

The 'CC' chemokine TARC/CCL17 was not detected in the AGS or MKN45 gastric epithelial cell lines, nor in any gastric biopsies. This novel chemokine which has previously been detected in bronchial epithelial cells was not expressed in gastric epithelial cells. In the bronchial epithelial cells, TARC/CCL17 was expressed by the bronchial epithelial cells and was up-regulated by the Th1 cytokine, IFN-γ and the Th2 cytokines IL-4 and IL-13 (Sekiya et al., 2000). TARC/CCL17 functions in a paracrine way to attract Th2 cells to the respiratory tract.

Two chemokines which have not been widely studied, were also found to be expressed in gastric mucosal biopsies. It has been proposed that both of these chemokines could be expressed constitutively to regulate homeostatic processes in the body (Mantovani, 1999). Gastric epithelial cell lines can express LARC/CCL20 constitutively, since it was detected in the AGS and MKN45 cell lines. Neither LARC/CCL20 nor BCA-1/CXCL13 were however detected in 'normal' gastric biopsies but they were present in patients with gastritis. This suggested that both could be induced in inflammation, but this was not dependent on the presence of *H. pylori*.

LARC/CCL20 expression was detected in most patients with gastritis. Expression was strong suggesting that the levels of this chemokine were high. In the small intestine and colon of the mice, where LARC/CCL20 is known to be expressed (Hieshima et al., 1997) the main function of LARC/CCL20 is to attract immature dendritic cells (Dieu et al., 1998) and T lymphocytes close to the epithelial surface to capture antigens in the intestinal lumen. The same process might be occuring in the stomach. LARC/CCL20 was also found to be expressed by mouse intestinal epithelial cells over Peyer's patches and mucosal lymphoid follicles in normal mice mucosa (Tanaka et al., 1999).

LARC/CCL20 probably plays a role in immunosurveillance in unstimulated epithelium. Since expression increases in gastritis, it appears to play a role in the inflammatory processes. LARC/CCL20 attracts CCR6-expressing memory T cells and immature dendritic cells (Dieu-Nosjean et al., 1999; Liao et al., 1999).

From the RT-PCR analysis of gastric biopsies, it cannot be determined whether it is epithelial cells that are predominantly expressing LARC/CCL20. Immunohistochemistry with an anti-LARC antibody was attempted on gastric biopsies. Since a suitable antibody was unavailable, it was not possible to identify which cells were expressing LARC/CCL20. Recently, Izadpanah et al. (2001) successfully stained inflamed colonic histologic specimens for LARC/CCL20. LARC/CCL20 was found to be minimally expressed in normal colonic epithelium but were markedly increased in epithelial cells in inflamed human colon. Izadpanah et al. (2001) also found constitutive expression of LARC/CCL20 in the HT-29, Caco-2, LS174T and I-407 colonic epithelial cell lines. LARC/CCL20 was up-regulated after these cell lines were stimulated with pro-inflammatory cytokines such as IL-1α or TNF-α. This indicated that LARC/CCL20 plays an important role in inflammation.

Similar to LARC/CCL20, BCA-1/CXCL13 was detected in some patients with gastritis but was not detected in samples from 'normal' patients. Expression was also not related to *H. pylori* infection in the small number of samples examined. However, BCA-1/CXCL13 expression was shown to be dependent upon *H. pylori* presence in the immunohistochemistry experiments.

BCA-1/CXCL13 has not previously been reported to be expressed by gastric epithelial cells and was not detected in stimulated AGS or MKN45 gastric epithelial cell lines. Previous studies have shown that in all biopsy samples from patients with *H. pylori*, BCA-1/CXCL13 is expressed by primary and secondary lymphoid follicles (Mazzucchelli et al., 1999).

BCA-1/CXCL13 plays a role in recruiting B cells. B lymphocytes can make antibodies which are important in eliminating foreign antigens and pathogens. (Abbas et al., 1997). This suggests it may be most important in bacterial infections. BCA-1/CXCL13 has also been found to be expressed in germinal centre-like structures of lymphoid aggregates in many patients with rheumatoid arthritis and in a few patients with osteoarthritis (Shi et al., 2001).

BCA-1/CXCL13 seems to be important in inflammation with less role in normal homeostasis, however, a study with larger numbers of patients would have to be studied to confirm this. The cell types expressing BCA-1/CXCL13 investigated by immunohistochemical analysis of gastric biopsy sections will be discussed in a later section.

To summarise, these results suggest that many chemokines play a role in inflammation associated with gastritis. Pro-inflammatory cytokines which are expressed in gastritis and inflammatory conditions in the gastric mucosa are increased during *H. pylori* infections. Although *H. pylori* has been associated with many pathological complications of the stomach, gastric inflammation due to other factors may also be important for chemokine expression. In this study, there were no obvious correlations between *H. pylori* with the mRNA expression of the other chemokines such as MIG/CXCL9, IP-10/CXCL10 and LARC/CCL20. This implied that these chemokines were more related to gastritis. These chemokines were probably expressed in the gastric mucosa to recruit inflammatory cells to the site of inflammation which can be caused by non-steroidal anti-inflammatory drug-induced gastritis or gastritis due to an over-production of gastric acid, but not *H. pylori*-associated gastritis.

None of the chemokines investigated in this study showed significant increase in expression in patients with *H. pylori*. The only possible exception is IL-8/CXCL8 which has previously been investigated (Moss et al., 1994; Yamaoka et al., 1995; Ando et al., 1996; Yamaoka et al., 1996; Kusugami et al., 1997).

The role of the chemokine BCA-1 in gastric disease and normal mucosal tissue

Immunohistochemical analysis of gastric biopsies clearly demonstrated that BCA-1/CXCL13 protein is expressed, therefore the BCA-1/CXCL13 mRNA detected by RT-PCR of biopsy samples was translated into chemokine protein *in vivo*.

Immunostaining with anti-human BCA-1/CXCL13 antibody detected this protein in sections taken from the gastric antrum in a 'normal' patient. Expression levels were low but positive staining was detected in a number of gastric epithelial cells in

gastric glands. The normal gastric cells could be expressing the BCA-1/CXCL13 chemokine as a homing chemokine which is needed for immunosurveillance of the gastric epithelial cells and tissues.

Some light positive staining for BCA-1/CXCL13 was observed in the lamina propria which consisted of inflammatory cells such as lymphocytes, macrophages and plasma cells.

It has previously been reported that gastric epithelial cells do not produce BCA-1/CXCL13 (Mazzucchelli et al., 1999). In the current study, the AGS and MKN45 gastric epithelial cell lines were also shown not to express BCA-1/CXCL13 even when stimulated in culture. The identification of the BCA-1/CXCL13 staining apparently in the gastric epithelium would need to be confirmed by analysis of larger numbers of biopsy samples from 'normal' patients. The presence of BCA-1/CXCL13 positive staining in the normal gastric epithelial cells in the current study could possibly be due to BCA-1/CXCL13 being expressed constitutively as a 'homing chemokine' for immunosurveillance of the gastric mucosa.

Since CXCR5, which is the chemokine receptor for BCA-1/CXCL13, may be constitutively expressed (faintly) by some gastric epithelial cells suggested by RT-PCR analysis of the MKN45 cells, BCA-1/CXCL13 detection may simply reflect its binding to CXCR5 expressed in the epithelium and this may contribute to their retention within the gastric mucosa (Mazzucchelli et al., 1999).

In the gastric antrum of the patient with non-*Helicobacter* associated gastritis, BCA-1/CXCL13 was stained strongly in the gastric tissues. RT-PCR analysis of similar samples also detected BCA-1/CXCL13 expression. The positive staining for BCA-1/CXCL13 was observed in some gastric epithelial cells in the gastric glands. In the antrum, BCA-1/CXCL13 stained positively in the upper region of the gastric glands with a little staining seen in the base of the gastric glands. The staining for BCA-1/CXCL13 was more dense in this patient than in the normal patient suggesting that more BCA-1/CXCL13 was induced as a result of the gastric inflammation associated with gastritis. Some BCA-1/CXCL13 was also expressed by inflammatory cells in the lamina propria. The increased expression of BCA-1/CXCL13 in gastritis suggests

that in addition to homeostasis, BCA-1/CXCL13 also has an inflammatory function. BCA-1/CXCL13 is known to be produced by B cells and it attracts memory T cells that express the receptor CXCR5 (Schaerli et al., 2000). CXCR5 was expressed by lymphocytes in lymphoid follicles and also in transformed B cells in 'MALT' lymphomas (Mazzucchelli et al.,1999). This retains the BCA-1/CXCL13 chemokine within the lymphoid follicle and 'MALT' lymphoma and makes the 'MALT' lymphoma less invasive compared to some other tumours.

In the section taken from the gastric body of a patient with *H. pylori* positive gastritis, there was dense staining for BCA-1/CXCL13 in the gastric epithelial cells in the majority of the gastric glands observed. The positive staining for BCA-1/CXCL13 was observed to be the more dense compared to the staining in *H. pylori*-negative patients with gastritis. There was also a lymphoid follicle in the slide which constitutes the mucosa-associated lymphoid tissue which was indicative of a chronic gastritis induced by *H. pylori* (Stolte et al., 1989; Genta et al., 1994; Parsonnet et al., 1994; Nakamura et al., 1998; Mazzucchelli et al., 1999; Hjelstrom, 2001). 'MALT' can disappear after eradication of the *H. pylori* infection, but in some instances, it can lead to the formation of gastric lymphomas (cancer).

The lymphoid follicle was stained very strongly for BCA-1/CXCL13 which suggests that BCA-1/CXCL13 was expressed by the B cells accumulating in the lymphoid follicle. This result is parallel to the findings of Mazzucchelli et al, (1999). B lymphocytes can make antibodies which are important in eliminating foreign antigens and bacterial pathogens, including *H. pylori*.

H. pylori does seem to play a role in increasing BCA-1/CXCL13 production in gastric tissues. BCA-1/CXCL13 could be expressed in order to eliminate *H. pylori* by the recruitment of antibodies by a Th1 reaction.

In summary, BCA-1/CXCL13 seems to play a small role in homing and immunosurveillance in normal gastric tissues but levels increase dramatically in gastritis tissues. This is possible as BCA-1/CXCL13 has both homeostatic and inflammatory functions and can be expressed constitutively or can be induced. *H. pylori* is a strong inducer of BCA-1/CXCL13 production which may be the reason *H.*

pylori infections can cause increased gastric cancers. Inflammation caused by factors other than *H. pylori* can also increase BCA-1/CXCL13 production as levels increase in gastritis.

Chemoattractants and their receptors are promising targets for treating inflammatory diseases. Blocking individual chemokines has proved to be surprisingly effective in suppressing a variety of immune reaction (Sallusto et al., 1998). BCA-1/CXCL13 or CXCR5 could be important targets to decrease inflammation which may also help to decrease cancer in the stomach.

CXCR4 expression in gastric epithelium

Immunohistochemical analysis clearly demonstrated that CXCR4 was expressed in 'real' gastric epithelial cells *in vivo*, since strong staining was detected at the surface of the mucosa in a normal patient. This suggested that CXCR4 was expressed constitutively in gastric epithelial cells. The expression of CXCR4 in 'normal' tissues indicates that it plays a role in gastric homeostasis.

CXCR4 expression was not restricted to epithelial cells but was also observed in inflammatory cells consisting of lymphocytes, plasma cells and macrophages in the lamina propria. CXCR4 has also been detected in endothelial cells (Gupta et al., 1998) and colonic epithelial cells (Jordan et al., 1999; Dwinell et al., 1999).

Examination of slides from 9 patients with *H. pylori* gastritis and 7 patients with non-*H. pylori*-associated gastritis showed very strong CXCR4 staining in the gastric epithelial cells of the gastric glands although more dense staining for CXCR4 was observed in the surface epithelial cells. This finding is different from the pattern of staining of BCA-1/CXCL13, suggesting that the chemokine receptor CXCR4 was mostly found in the surface epithelial cells of the gastric glands. Interestingly, Jordan et al. (1999) found that more dense staining for CXCR4 appeared at the base of the colonic glands and crypts. Therefore Jordan et al. (1999) hypothesised that SDF-1α/CXCL12 and CXCR4 were important for colonic epithelial cell renewal and maintenance. In the stomach, CXCR4 may play a different role, which requires for CXCR4 to be expressed on the surface of the epithelial cells, such as in defence.

More positive staining was observed in the patients with *H. pylori*-associated gastritis compared to the patients with non-*H. pylori*-associated gastritis and normal patients. CXCR4 may have a physiologic role in gastric epithelial cell homeostasis and appears to be up-regulated in gastritis. *H. pylori* may play a role in up-regulating the expression of CXCR4 which may then activate the gastric epithelial cells to launch an immunological response against the bacteria. More studies would have to be performed to examine the CXCR4 for any other function.

In a patient with adenocarcinoma, CXCR4 was clearly stained in epithelial cells which looked abnormal. However, there was no obvious differences in the density of the staining from the 'normal' (non cancerous) cells surrounding the tumour. Tachibana et al. (1998) found that CXCR4 was important for the development of mice gastrointestinal vasculature *in utero*. The development of the gastrointestinal vasculature might be important if extrapolated to the development of gastric cancers and lymphomas. It is assumed that good vasculature was needed for cancer growth. However, the role of CXCR4 in gastric cancer is speculative as Mitra et al. (1999) found that there was no difference in the expression of CXCR4 mRNA between gastric, colon and squamous oesophageal cancers compared to normal tissues. This was also demonstrated in the current study. SDF-1α/CXCL12 was found to be decreased or not expressed in cancer tissues including gastric cancers (Shibuta et al., 1997). This suggests that the signalling via the SDF-1α/CXCR4 pathway is reduced in cancer. Therefore, CXCR4 might just have a homeostatic role in the development of the gastric vasculature in the stomach. In other cancerous tissue, like the breast, CXCR4 is up-regulated and is believed to induce invasive responses (Strieter, 2001) and possibly to stimulate angiogenesis and tumour vascularization. It is not known whether CXCR4 levels increase in gastric cancer and increase metastasis. Further studies would be required to ascertain the role of CXCR4 in gastric cancer.

There is still plenty more to study regarding how chemokines expressed as a result of *H. pylori* infections generate gastric mucosa damage as the mechanisms are still not fully understood. *H. pylori* infection is responsible for initiating Th1 type of reaction by the epithelial cells. An inadequate Th2 response leads to failure of the host to eradicate the bacteria (Wilson et al., 1999). Therefore, epithelial cells only play a

secondary role in the pathogenesis of disease, which is more related to the final histopathology of the disease instead of the initiation of gastric lesions (Strober, 1998). Other than the cytokine/chemokine secretion of epithelial cells in response to *H. pylori* infection, signalling pathways are important in initiating gastric mucosa damage. An example of this is the ability of *H. pylori* to induce gastric epithelial apoptosis which is achieved by direct contact between the bacteria and the epithelial cells and expression of the Bcl-2 family protein called Bak (Chen, et al., 1997).

5.1 FUTURE WORK

1. It would be interesting to obtain a larger number of gastric biopsy samples from the antrum and gastric body of patients with different pathological conditions such as gastritis with *H. pylori*, gastritis without *H. pylori*, normal, gastric ulcers, gastric cancer/adenocarcinoma and MALT lymphoma. Novel chemokine expression would be investigated. This work could be conducted in parallel with the AGS and MKN45 gastric epithelial cell lines to determine if these chemokines detected could potentially be expressed by gastric epithelial cells in the gastric biopsies.

2. If possible, surgical specimens of the stomach could be obtained to enable larger amounts of gastric tissue to be harvested for isolation of gastric epithelial cells. This would possibly overcome the problem of poor isolated epithelial cell yield from biopsies. The isolated epithelial cells maintained in culture would provide information about chemokine expression and secretion and chemokine receptor expression by epithelial cells alone.

3. The chemokine receptors that would be useful to study on the gastric epithelial cells would be CCR6 which is the receptor for LARC/CCL20 and CXCR5, which is the receptor for BCA-1/CXCL13. This would compliment the studies already carried out which have indicated that these chemokines are expressed in the gastric mucosa. This would be very interesting particularly since CCR6 is known to be abundantly expressed by normal colonic epithelial cells (Izadpanah et al., 2001).

4. *H. pylori* could be cultured and added to a culture of gastric epithelial cell lines *in vitro* to compare which chemokines are induced by *H. pylori*. These results could be compared to those obtained from the analysis of biopsies from *H. pylori*-infected patients.

5. Once a suitable antibody for anti-human LARC/CCL20 antibody for immunohistochemistry is available, the gastric histological slides could be stained for LARC/CCL20 to determine whether its levels are increased in gastritis.

There is a great deal still unknown about the role of chemokines in the gastric mucosa. Studies need to be conducted to find a link between the chemokines induced by *H. pylori* and carcinogenicity in the stomach. So far, BCA-1/CXCL13 has been linked with MALT-lymphoma. The possible connection between chemokines and other cancer pathways might also be worth investigating as a full understanding of chemokines and their mechanisms of action may be extremely useful in therapeutic treatments of clinical diseases of the stomach in the future.

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